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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 :
C07K 5/04, 7/04, 7/06, 7/08, 14/00, 14/435, 14/705, G01N 33/53, 33/567

A1

(11) International Publication Number: **WO 97/30074**

(43) International Publication Date: 21 August 1997 (21.08.97)

(21) International Application Number: PCT/US97/02298

(22) International Filing Date: 14 February 1997 (14.02.97)

(30) Priority Data:
08/602,999 16 February 1996 (16.02.96) US

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(81) Designated States: AL, AM, AU, AZ, BA, BB, BG, BR, BY, CA, CN, CU, CZ, EE, GE, HU, IL, IS, JP, KG, KP, KR, KZ, LC, LK, LR, LT, LV, MD, MG, MK, MN, MX, NO, NZ, PL, RO, RU, SG, SI, SK, TJ, TM, TR, TT, UA, UZ, VN, YU, ARIPO patent (KE, LS, MW, SD, SZ, UG), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: ISOLATION AND USE OF SH3 BINDING PEPTIDES

(57) Abstract

Peptides having general and specific binding affinities for the Src homology region 3 (SH3) domains of proteins are disclosed in the present invention. In particular, SH3 binding peptides have been isolated from phage-displayed random peptide libraries which had been screened for isolates that bind to bacterial fusion proteins comprising SH3 and glutathione S-transferase (GST). Preferred peptides are disclosed which comprise a core 7-mer sequence (preferably, a consensus motif) and two or more, preferably at least six, additional amino acid residues flanking the core sequence, for a total length of 9, preferably at least 13, amino acid residues and no more than about 45 amino acid residues. Such peptides manifest preferential binding affinities for certain SH3 domains. The preferred peptides exhibit specific binding affinities for the Src-family of proteins. *In vitro* and *in vivo* results are presented which demonstrate the biochemical activity of such peptides.

BNSDOCID: <WO___9730074A1_L>

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ISOLATION AND USE OF SH3 BINDING PEPTIDES

1. Field of the Invention

The present invention relates to SH3 binding peptides having a broad range of binding specificities. That is, certain members of the SH3 binding peptides disclosed bind with approximately the same facility with SH3 domains derived from different SH3 domain-containing proteins. Other members, in contrast, bind with a much greater degree of affinity for specific SH3 domains. The SH3 binding peptides are obtained from random peptide libraries that are also phage-displayed. Methods are described of obtaining the phage clones that bind to the SH3 domain targets and of determining their relevant nucleotide sequences and consequent primary amino acid sequence of the binding peptides. The resulting SH3 binding proteins are useful in a number of ways, including, but not limited to, providing a method of modulating signal transduction pathways at the cellular level, of modulating oncogenic protein activity or of providing lead compounds for development of drugs with the ability to modulate broad classes, as well as specific classes, of proteins involved in signal transduction.

2. Background of the Invention

2.1. Src and the SH3 Domain

Among a number of proteins involved in eukaryotic cell signaling, there is a common sequence motif called the SH3 domain. It is 50-70 amino acids in length, moderately conserved in primary structure, and can be present from one to several times in a large number of proteins involved in signal transduction and in cytoskeletal proteins.

The protein pp60*c-src* represents a family of at least nine non-receptor protein tyrosine kinases (NR-PTKs). Members of this family share an overall structural organization comprising a series of catalytic and non-catalytic domains. In Src, a 14-amino-acid myristylation

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signal resides at the extreme amino-terminus, and is followed by a unique region that is not highly conserved among family members. Following this region are two highly conserved 60- and 100-amino-acid regions, the Src homology (SH) domains 3 and 2, respectively. SH2 and SH3 domains have been shown to play an important role in mediating protein-protein interactions in a variety of signaling pathways. Koch, C.A., et al., in Science (1991) 252:668-74. The carboxy-terminal half of Src contains the PTK catalytic domain, as well as a negative regulatory tyrosine (Y527) near the carboxy terminus. Phosphorylation of this residue (e.g., by Csk) results in the inhibition of PTK activity. Cooper, J.A., et al., in Science (1986) 231:1431-1434. Mutation of Y527->F generates forms of Src with increased PTK and oncogenic activity. Cartwright, C.A., et al., in Cell (1987) 49:83-91; Kmiecik, T.E., et al., in Cell (1987) 49:65-73; and Piwicna-Worms, H., et al., in Cell (1987) 75-82.

The fact that some mutations which result in increased Src PTK and transforming activity map to the Src SH2 (Seidel-Dugan, C., et al., in Mol. Cell. Biol. (1992) 12:1835-45; and Hirai, H. and Varmus, H.E. in Mol. Cell. Biol. (1990) 10:1307-1318) and SH3 domains (Seidel-Dugan, C., et al., supra; Hirai, H. and Varmus, H.E., *supra*; Superti-Furga, G., et al., in Embo. J. (1993) 12:2625-34; and Potts, W.M., et al., in Oncogene Res. (1988) 3:343-355) suggests a negative regulatory role for these domains. That phosphotyrosine residues within specific sequence contexts represent high affinity ligands for SH2 domains suggests a model in which the SH2 domain participates in Y527-mediated inhibition of PTK activity by binding phosphorylated Y527, thereby locking the kinase domain in an inactive configuration. Matsuda, M., Mayer, B.J., et al., in Science (1990) 248:1537-1539. This model is supported by the observation that phosphopeptides corresponding to the carboxy-terminal tail of Src bind active, but not inactive, variants of Src. Roussel, R.R., et al., in Proc. Natl. Acad. Sci. U S A (1991) 88:10696-700; and Liu, X., et al., in Oncogene (1993) 8:1119-1126.

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The mechanism of SH3-mediated inhibition of Src PTK activity remains unclear. There is evidence that pY527-mediated inhibition of Src PTK activity involves the SH3 domain as well as the SH2 domain. Okada, M., Howell, et al., in J. Biol. Chem. (1993) 268:18070-5; Murphy, S.M., et al., in Mol. Cell. Biol. (1993) 13:5290-300; and Superti-Furga, G., et al., *supra*. Although these effects are thought to be a consequence of SH3-mediated protein-protein interactions, precisely how the Src SH3 domain exerts its negative regulatory effect is unclear. Identification of high affinity ligands for the Src SH3 domain could help resolve these issues.

2.2. Protein Tyrosine Kinases and The Immune Response

Src-related tyrosine kinases are expressed in a variety of cell types including those of the immune system (lymphocytes, T cells, B cells, and natural killer cells) and the central nervous system (neural cells, neurons, oligodendrocytes, parts of the cerebellum, and the like). Umemori, H. et al., in Brain Res. Mol. Brain Res. (1992) Dec. 16(3-4):303-310. Their presence in these cells and tissues and their interaction with specific cell surface receptors and immunomodulatory proteins (such as T cell antigen receptor, CD14, CD2, CD4, CD40 or CD45) suggest that these kinases serve an important role in the signalling pathways of not only the central nervous system but of the immune system, as well. See, e.g., Ren, C.L. et al., in J. Exp. Med. (1994) 179(2):673-680 (signal transduction via CD40 involves activation of Lyn kinase); Donovan, J.A. and Koretzky, G.A., in J. Am. Soc. Nephrol. (1993) 4(4):976-985 (CD45, the immune response, and regulation of Lck and Fyn kinases); and Carmo, A.M. et al., in Eur. J. Immunol. (1993) 23(9):2196-2201 (physical association of the cytoplasmic domain of CD2 with p561ck and p59fyn).

For instance, mice with disruptions in their Src-like genes, Hck and Fgr, possess macrophages with impaired phagocytic activity or exhibit a novel immunodeficiency

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characterized by an increased susceptibility to infection with Listeria monocytogenes. Lowell, C.A. et al., in Genes Dev. (1994) 8(4):387-398. Also, it has been shown that bacterial lipopolysaccharide (LPS) activates CD14-associated p56lyn, p68hck, and p59c-fgr, while inducing the production of lymphokines, such as TNF-alpha, IL-1, IL-6, and IL-8. Inhibition of the protein tyrosine kinases blocks production of TNF-alpha and IL-1.

2.3. SH3 Binding Peptides

As mentioned above, it has long been suspected that SH3 domains are sites of protein-protein interaction, but it has been unclear what SH3 domains actually bind. Efforts to identify ligands for SH3 domains have led to the characterization of a number of SH3-binding proteins, including 3BP1 and 2 (Ren, R., Mayer, et al., in Science (1993) 259:1157-61), SOS (Olivier, J.P., et al., in Cell (1993) 73:179-91; and Rozakis-Adcock, M., et al., in Nature (1993) 363:83-5), p85 PI-3' Kinase (Xingquan, L., et al., in Mol. Cell. Biol. (1993) 13:5225-5232), dynamin (Gout, 1., et al., in Cell (1993) 75:25-36), AFAP-110 (Flynn, D.C., et al., in Mol. Cell. Biol. (1993) 13:7892-7900), and CD42 (Barfod, E.T., et al., in J. Biol. Chem. (1993) 268:26059-26062). These proteins tend to possess short, proline-rich stretches of amino acids, some of which have been directly implicated in SH3 binding. A variety of consensus sequences have been proposed, although the similarity among proline-rich regions of different SH3-binding proteins tends to be fairly low. Also, attempts to build consensus sequences are likely complicated by the incorporation of data from proteins that bind different SH3 domains.

Thus, Cicchetti, P., et al., in Science (1992) 257:803-806, published their work relating to the isolation and sequencing of two naturally-occurring proteins that could be bound *in vitro* by the SH3 domain of the *abl* oncogene product. These workers found that SH3 domains bind short, proline-rich regions of such proteins. Subsequently, this same group

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disclosed further results (Ren, R. et al., *supra*) in which the SH3 binding sites of the SH3 binding proteins were localized to "a nine- or ten-amino acid stretch rich in proline residues." A consensus sequence incorporating the features of the SH3 binding sites of four SH3 binding proteins was proposed: XPXXPPP¥XP (SEQ ID NO:1), wherein X indicates a position in the amino acid sequence which is not conserved among the four SH3 binding proteins, P represents proline, and ¥ indicates a hydrophobic amino acid residue, such as P or L.

The screening of complex random peptide libraries has been used to identify peptide epitopes for monoclonal (Scott, J.K. and Smith, G.P. in Science (1990) 249:386-390) and polyclonal (Kay, B.K., et al., in Gene (1993) 128:59-65) antibodies, as well as peptide ligands for a variety of proteins, including streptavidin (Devlin, J.J., et al., in Science (1990) 249:404-406; and Lam, K., et al., in Nature (1991) 354:82-84), the endoplasmic reticulum chaperone BiP (Blond-Elguindi, S., et al., in Cell (1993) 75:717-728), and CaM (Dedman, J.R., et al., in J. Biol. Chem. (1993) 268:23025-23030).

Recently, Chen, J.K. et al., in J. Am. Chem. Soc. (1993) 115:12591-12592, described ligands for the SH3 domain of phosphatidylinositol 3-kinase (PI-3′ Kinase) which were isolated from a biased combinatorial library. A "biased" library is to be distinguished from a "random" library in that the amino acid residue at certain positions of the synthetic peptide are fixed, i.e., not allowed to vary in a random fashion. Indeed, as stated by these research workers, screening of a "random" combinatorial library failed to yield suitable ligands for a PI-3′ Kinase SH3 domain probe. The binding affinities of these unsuitable ligands was described as weak, >100 μM, based on dissociation constants measured by the Biosensor System (BIAcore).

More recently, Yu, et al. (Yu, H., et al., in Cell (1994) 76:933-945) used a "biased" synthetic peptide library of the form XXXPPXPXX (SEQ ID NO:2), wherein X represents any

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amino acid other than cysteine, to identify a series of peptides which bind the Src and PI-3′ Kinase SH3 domains. The bias was accomplished by fixing the proline residues at the specific amino acid positions indicated for the "random" peptide. As stated previously, without this bias, the technique disclosed fails to identify any SH3 domain-binding peptides.

A consensus sequence, based on 13 binding peptides was suggested: RXLPPRPXX (SEQ ID NO:3), where X tends to be a basic residue (like R, K or H). The binding affinities of several SH3 binding peptides were disclosed as ranging from 8.7 to 30 μM. A "composite" peptide, RKLPPRPRR (SEQ ID NO:4), was reported to have a binding affinity of 7.6 μM. This value compares favorably to the binding affinity of the peptide, VPPPVPPRRR (SEQ ID NO:5), to the N-terminal SH3 domain of Grb2. See, Kraulis, P.J. J. Appl. Crystallogr. (1991) 24:946. Recognizing the limitations of their technique, Chen and co-workers, *supra*, stated that their results "illustrate the utility of biased combinatorial libraries for ligand discovery in systems *where there is some general knowledge of the ligand-binding characteristics of the receptor*" (emphasis added).

Yu and co-workers, *supra*, further described an SH3 binding site consensus sequence, XpØPpXP (SEQ ID NO:6), wherein X represents non-conserved residues, Ø represents hydrophobic residues, P is proline, and p represents residues that tend to be proline. A consensus motif of RXLPPRPXX (SEQ ID NO:7), where X represents any amino acid other than cysteine, was proposed for ligands of PI-3′ Kinase SH3 domain. A consensus motif of RXLPPLPRϕ (SEQ ID NO:8), where ϕ represents *hydrophobic* residues, was proposed for ligands of Src SH3 domain. Still, the dissociation constants reported for the 9-mer peptides ranged only from about 8-70 μM and selectivity between one type of SH3 domain and another was relatively poor, the K_Ds differing by only about a factor of four.

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Hence, there remains a need to develop techniques for the identification of Src SH3 binding peptides which do not rely on such "biased" combinatorial peptide libraries that are limited to a partially predetermined set of amino acid sequences. Indeed, the isolation of SH3 binding peptides from a "random" peptide library has not been achieved successfully before now. Furthermore, particular peptides having much greater binding affinities, whether general or more selective binding for specific SH3 domains, remain to be identified. Binding peptides specific for particular SH3 domains are useful, for example, in modulating the activity of a particular SH3 domain-containing protein, while leaving others bearing an SH3 domain unaffected. Still, the more promiscuous general binding peptides are useful for the modulation of a broad spectrum of SH3 domain-containing proteins.

The present invention relates to such SH3 binding peptides, methods for their identification, and compositions comprising same. In particular, peptides comprising particular sequences of amino acid residues are disclosed which were isolated from random peptide libraries. In the present invention, clones were isolated from a phage-displayed random peptide library which exhibited strong binding affinities for SH3 domain-containing protein targets. Some of these protein targets, include Abl, Src, Grb2, PLC-δ, PLC-γ, Ras GAP, Nck, and p85 PI-3' Kinase. From the nucleotide sequence of the binding phage, the amino acid sequence of the peptide inserts has been deduced. Synthetic peptides having the desired amino acid sequences are shown to bind the SH3 domain of the target proteins. In particular, synthetic peptides combining a core consensus sequence and additional amino acid residues flanking the core sequence are especially effective at binding to particular target protein SH3 domains. The SH3 binding peptides disclosed herein can be utilized in a number of ways, including the potential modulation of oncogenic protein activity *in vivo*. These peptides also serve as useful leads in the production of

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peptidomimetic drugs that modulate a large class of proteins involved in signal transduction pathways and oncogenesis.

3. Summary of the Invention

Accordingly, three phage-displayed random peptide libraries were screened for isolates that bind to bacterial fusion proteins consisting of the Src homology region 3 (SH3) and glutathione S-transferase (GST). DNA sequencing of the isolates showed that they contained sequences that resemble the consensus motif, RPLPPLP (SEQ ID NO:9), within their 8, 22, or 36 amino acid long random regions. When peptides were synthesized corresponding to the pIII inserts of the SH3-binding phage, they bound to the GST fusions of the SH3 domains of Src and the Src-related proteins, such as Yes, but not of Grb2, Crk, Abl, or PLCγ1. The synthesized peptides bind quite well to the Src SH3 domain and act as potent competitors of natural Src SH3 interactions in cell lysates. For instance, these peptides can compete with radiolabelled proteins from cell lysates in binding to immobilized Src-GST, with an apparent IC_{50} of 1-10 μM. When a peptide, bearing the consensus sequence RPLPPLP (SEQ ID NO:9) was injected into *Xenopus laevis* oocytes, it accelerated the rate of progesterone-induced maturation. These results demonstrate the utility of phage-displayed random peptide libraries in identifying SH3-binding peptide sequences and that such identified peptides exhibit both *in vivo* and *in vitro* biological activity.

Thus, it is an object of the present invention to provide peptides having at least nine and up to forty-five amino acid residues, including an amino acid sequence of the formula, R-2-L-P-5-6-P-8-9 (SEQ ID NO:10), positioned anywhere along the peptide, in which each number represents an amino acid residue, such that 2 represents any amino acid residue except cysteine, 5 and 6 each represents a hydrophobic amino acid residue, 8 represents any amino acid residue except cysteine, and 9 represents a hydrophilic amino acid residue except cysteine, each letter being the standard

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one-letter symbol for the corresponding amino acid, said peptide exhibiting a binding affinity for the SH3 domain of Src, provided that said peptide is not R-P-L-P-P-L-P-T-S (SEQ ID NO:11). In a particular embodiment of the present invention, the peptides also exhibit a binding affinity for the SH3 domain of Src-related proteins, including Yes, Fyn, Lyn, Lck, Hck and Fgr.

The present invention also contemplates SH3 domain-binding peptides that further comprise a C-terminal-flanking amino acid sequence of the formula 10, 10-11, 10-11-12, 10-11-12-13 (SEQ ID NO:12) or 10-11-12-13-14 (SEQ ID NO:13), in which each number represents any amino acid residue except cysteine, such that 10 is bound to 9 by a peptide bond. Furthermore, peptides are also provided which further comprise an N-terminal-flanking amino acid sequence of the formula 1', 2'-1', 3'-2'-1' or 4'-3'-2'-1' (SEQ ID NO:14) in which each number represents any amino acid residue except cysteine, such that 1' is bound to R by a peptide bond.

Thus, in a particular embodiment, a peptide is disclosed having at least thirteen and up to forty-five amino acid residues, including an amino acid sequence of the formula, 3'-2'-1'-R-2-L-P-5-6-P-8-9-10 (SEQ ID NO:15), positioned anywhere along the peptide, in which each number represents an amino acid residue, such that 3', 2', 1', 2, 8, and 10 each represents any amino acid residue except cysteine, 5 and 6 each represents a hydrophobic amino acid residue, and 9 represents a hydrophilic amino acid residue except cysteine, each letter being the standard one-letter symbol for the corresponding amino acid, said peptide exhibiting a binding affinity for the SH3 domain of Src.

The present invention also seeks to provide new consensus sequences or motifs that reflect variations in SH3 domain binding selectivities or specificities. The present invention also contemplates conjugates of the SH3 binding peptides and a second molecule or chemical moiety. This second molecule may be any desired substance whose delivery to the region of the SH3 domain of a particular protein (or

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cell containing the protein) is sought. Possible target cells include, but are not limited to, neural cells, immune cells (e.g., T cells, B cells, natural killer cells, and the like), osteoclasts, platelets, epidermal cells, and the like, which cells express Src, Src-related proteins, and potentially, other SH3 domain-containing proteins. In this manner, the modulation of the biological activity of proteins bearing an SH3 domain can be accomplished.

Other methods and compositions consistent with the objectives of the present invention are likewise disclosed. In particular, a method is disclosed of modulating the activity of Src or Src-related proteins comprising administering a composition comprising an effective amount of a peptide of the present invention and a carrier, preferably a pharmaceutically acceptable carrier. In a specific embodiment, the contemplated method results in the inhibition of the activity of Src or Src-related proteins. Alternatively, the method is effective to activate Src or Src-related proteins.

In yet another embodiment, a method is disclosed of identifying a peptide having a region that binds to an SH3 domain comprising: (a) providing an immobilized target protein comprising an SH3 domain; (b) incubating the immobilized target protein with an aliquot taken from a random peptide library; (c) washing unbound library peptides from the immobilized target protein; (d) recovering the peptide bound to the immobilized target protein; and (e) determining the primary sequence of the SH3 domain-binding peptide.

Moreover, a method is disclosed of imaging cells, tissues, and organs in which Src or Src-related proteins are expressed, which comprises administering an effective amount of a composition comprising an SH3 domain-binding peptide conjugated to detectable label or an imaging agent.

Other objectives of the present invention will become apparent to one of ordinary skill in the art after

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consideration of the above disclosure and the following detailed description of the preferred embodiments.

The invention also provides assays for identifying a compound that affects the binding between a first molecule comprising an SH3 domain and a second molecule that binds to the SH3 domain comprising incubating one or more candidate compounds from which it is desired to select such a compound with the first molecule and the second molecule under conditions conducive to binding and detecting the one or more compounds that affect binding of the first molecule to the second molecule.

Also provided are kits for performing such assays comprising a first molecule comprising an SH3 domain and a second molecule that binds to the SH3 domain.

4. Brief Description of the Figures

FIG. 1 illustrates a scheme for the generation of a random 36 amino acid peptide library (TSAR-9; e.g., SEQ ID NO:16). Oligonucleotides were synthesized (SEQ ID NOS:17-18), converted into double-stranded DNA, cleaved with restriction enzymes (SEQ ID NOS:19-20), and cloned into the M13 vector, m663. The random peptide region encoded by the oligonucleotides is shown in the box (SEQ ID NO:16) and is situated at the N-terminus of mature protein III (SEQ ID NO:21). SEQ ID NO:22 includes the three amino acids preceding the signal peptidase cleavage site.

FIG. 2 illustrates a scheme for the generation of a random 22 amino acid peptide library (TSAR-12; e.g., SEQ ID NO:23). Oligonucleotides were synthesized (SEQ ID NOS:24-25), converted into double-stranded DNA, cleaved with restriction enzymes (SEQ ID NOS:26-27), and cloned into the M13 vector, m663. The random peptide region encoded by the oligonucleotides is shown in the box (SEQ ID NO:23) and is situated at the N-terminus of mature protein III (SEQ ID NO:28). SEQ ID NO:29 includes the three amino acids preceding the signal peptidase cleavage site.

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FIG. 3 illustrates a scheme for the generation of a random 8 amino acid peptide library (R8C; SEQ ID NO:30). Oligonucleotides were synthesized (SEQ ID NOS:31-32), converted into double-stranded DNA, cleaved with restriction enzymes (SEQ ID NOS:33-34), and cloned into the M13 vector, m663. The random peptide region (SEQ ID NO:30) is flanked by cysteine residues and is situated at the N-terminus of mature protein III (SEQ ID NO:35).

FIG. 4 illustrates the possible origin of one class of double-insert R8C recombinants (e.g., encoding SEQ ID NO:36). Double-stranded oligonucleotides (e.g., SEQ ID NO:37) may have ligated in a head-to-head fashion at the *Xba* I site prior to cloning in the *Xho* I- *Xba* I cleaved M13 vector.

FIG. 5 shows a list of random peptide recombinants (SEQ ID NOS:38-61 and 106) isolated by the method of the present invention and the displayed peptide sequence. The amino acid sequences are aligned to highlight the core sequences. The flanking sequences are shown to the N-terminal and C-terminal ends of the core sequence. SEQ ID NOS:38-61 are shown in order from top to bottom except that SSCDHTLGLGWCGSRSTRQLPIPP TTTRPSR is SEQ ID NO:106 and RPLPPLP is SEQ ID NO:9. T12.Src3.1 is a Class II ligand (See Section 6.14.5).

FIG. 6 graphically illustrates the relative binding affinities of selected phage clones for various SH3 domains. The results indicate that certain amino acid sequences provide generic SH3 domain binding, while others can provide greater selectivity for the SH3 domain of Src. Still other clones exhibit Src SH3 domain preferential binding.

FIG. 7 shows the binding of synthetic peptides (SEQ ID NOS:9 and 62-70) representing Src SH3-selected phage inserts to Src SH3-GST fusion target (shaded columns) over background GST binding (unshaded columns) relative to the core peptide RPLPPLP (SEQ ID NO:9) and proline-rich peptide segments derived from naturally occurring proteins. Bound biotinylated peptide was detected with streptavidin-alkaline phosphatase ELISA. Each point was performed in triplicate; average absorbance at 405 nm is presented. Error bars

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represent SD. SEQ ID NOS:62-70 are shown in order from top to bottom except that RPLPPLP is SEQ ID NO:9.

FIG. 8 illustrates the relative specificity of selected peptides (SEQ ID NOS:9 and 62-70) for SH3 domains derived from different proteins. In particular, the binding affinities of the peptides for the SH3 domains of the following protein fusion targets were tested: Src SH3-GST, Yes SH3-GST, Grb2-GST, Crk SH3-GST, Abl SH3-GST, PLCγ1 SH2SH3-GST. Bound biotinylated peptide was detected with streptavidin-alkaline phosphatase. Each point was performed in triplicate; values are average signal (absorbance at 405 nm) above GST background, with error bars representing standard deviation. Hatched bars indicate saturation of the ELISA signal. SEQ ID NOS:62-70 are shown in order from top to bottom except that RPLPPLP is SEQ ID NO:9.

FIG. 9 presents the results of competition experiments in which selected peptides were found to inhibit the binding of proteins from cell lysates to immobilized Src SH3-GST or Abl SH3-GST protein fusion targets.

FIG. 10 presents a graph illustrating the increased rate of progesterone-induced maturation of oocytes injected with an SH3 domain-binding peptide, VLKRPLPIPPVTR (SEQ ID NO:64), of the present invention. Briefly, Stage VI oocyted were prepared and injected as previously described (see, Kay, B.K., in Methods in Cell Biol. (1991) 36:663-669). Oocytes were injected with 40 nL of 100 μM test peptide or water. After injection, the oocytes were placed in 2 μg/mL progesterone (Sigma, St. Louis, MO) and scored hourly for germinal vesicle breakdown (GVBD). LAPPKPPLPEGEV is SEQ ID NO:70.

FIG. 11 shows the results of fluorescence experiments in which certain peptides, Panel A = VLKRPLPIPPVTR (SEQ ID NO:64), Panel B = GILAPPVPPRNTR (SEQ ID NO:63), Panel C = RSTPRPLPPLPTTR (SEQ ID NO:67), of the invention were shown to localize within cellular compartments thought to contain Src or Src-related proteins.

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FIG. 12 illustrates a scheme for the generation of a biased peptide library. Oligonucleotides were synthesized (SEQ ID NOS:162-163), converted into double-stranded DNA (SEQ ID NO:454), cleaved with restriction enzymes XhoI and XbaI (SEQ ID NOs:455-456), and cloned into the mBAX vector (SEQ ID NOs:457-458), described further below in the Examples section. The biased peptide region (SEQ ID NO:459) is situated at the N-terminus of mature pIII protein. CTAGACGTGTCAGT is a portion of SEQ ID NO:162. ACTGACACGT is a portion of SEQ ID NO:454. TCGAGGCACAG is a portion of SEQ ID NO:454.

FIG. 13 illustrates the peptide sequence encoded in the mBAX vector situated at the N-terminus of mature pIII protein. TCCTCGAGTATCGACATGCCTTAGACTGCTAGCACTATGTACAACATGCTT CATCGCAACGAGCCA is SEQ ID NO:460. SSIDMP*TASTMYNM LHRNEP is SEQ ID NO:461. GGTGGGAGGAAGTTGAGCCCGCCCGCCAACGA CATGCCGCCCGCCCTCCTGAAGAGGTCTAGA is SEQ ID NO:462. GGRKLSPPANDMPPALLKRSR is SEQ ID NO:463.

FIG. 14 illustrates the relative binding of SH3-selected phage clones to various SH3 domains. Two clones (A and B) representing each consensus motif were assayed for binding to 1 μg of each immobilized GST-SH3 fusion protein. Bound phage were detected by anti-phage ELISA. Sequences of peptides displayed by each clone are aligned with their respective consensus motifs. Invariant proline residues are underlined. Solid bars, specific binding; open bars, cross-reactive binding. Values are average OD_{405} ± SD (N =3).

5. Detailed Description of the Invention

5.1. General Considerations

The present invention relates to peptides that exhibit a binding affinity for an SH3 domain, which domain has been found to be present in a number of physiologically significant proteins. In particular, peptides are disclosed which exhibit general binding characteristics to the SH3 domains found in a group of proteins, including but not limited to Abl, Src, Grb2, PLC-δ, PLC-γ, Ras GAP, Nck, and

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p85 PI-3' Kinase. Preferred peptides exhibit selective, if not specific, binding affinity for the SH3 domain of Src. As described herein, the peptides of the present invention include a core sequence, preferably a consensus seqeunce, and additional amino acid residues that flank the core sequence. These peptides, including the methods for their identification, are described in greater detail, below.

Thus, in a specific embodiment of the invention, peptides are provided which have at least nine and up to about forty-five amino acid residues, including an amino acid sequence resembling the formula,

R-2-L-P-5-6-P-8-9 (SEQ ID NO:10),

positioned anywhere along the peptide. In the above-mentioned formula, each number represents an amino acid residue, such that 2 represents any amino acid residue except cysteine, 5 and 6 each represents a hydrophobic amino acid residue, 8 represents any amino acid residue except cysteine, and 9 represents a hydrophilic amino acid residue except cysteine. Each letter used in the formulas herein represent the standard one-letter symbol for the corresponding amino acid. When the peptide is a 9-mer, the peptide R-P-L-P-P-L-P-T-S (SEQ ID NO:11) is excluded. The peptides of particular interest are those that exhibit a binding affinity for the SH3 domain of Src and Src-related proteins, including Yes, Fyn, Lyn, Lck, Hck and Fgr. Preferably, the peptides of the invention exhibit a binding affinity for the SH3 domain of Src, which is at least three-fold, more preferably at least four-fold, most preferably at least about five-fold greater than that exhibited by the peptide RPLPPLP (SEQ ID NO:9). In still other embodiments, the peptides exhibit a binding affinity for the SH3 domain of Src which is at least ten-fold greater than that exhibited by the peptide RPLPPLP (SEQ ID NO:9).

In specific embodiments, peptides are disclosed in which the various amino acid residues at the indicated positions may independently have the following preferred identities: 2 is a P, R, A, L, Q, E or S, more preferably P or R; 5

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represents a P, M, I or L, more preferably P or M; 6 is a P, L, I or V, more preferably P or L; 8 is a T, R, P, I, N, E, V, S, A, G or L, more preferably T or R; and 9 is a T, R, S, H or D, more preferably T or R. Despite the preference for hydrophobic amino acid residues at 5 and 6, in some cases it may be desirable to have hydrophilic amino acid residues at these positions. Specifically, amino acid residue 5 may be a T, R or S, and amino acid residue 6 may be a T or R. Likewise, while a hydrophilic amino acid residue is preferred at position 9, in some instances a hydrophobic residue, such as a P or A, may be desirable.

The present invention also contemplates SH3 domain-binding peptides with a minimum length of 10, 11, 12, 13, 14, 15 or more amino acids. Such peptides contain additional amino acid residues flanking the core sequence of R-2-L-P-5-6-P (SEQ ID NO:71) either at the C-terminal end, the N-terminal end or both. Thus, for example, such peptides include those that further comprise a C-terminal-flanking amino acid sequence of the formula 10, 10-11, 10-11-12, 10-11-12-13 (SEQ ID NO:12) or 10-11-12-13-14 (SEQ ID NO:13), in which each number represents any amino acid residue except cysteine, such that the amino acid residue 10 is bound to the amino acid residue 9 by a peptide bond. In that case, specific embodiments include an amino acid residue 10 which is T, R, L, S, D, P, A or N, preferably T or R, an amino acid residue 11 which is R, P, A, Q, S or T, preferably R or P, an amino acid residue 12 which is P, S, R or T, preferably P or S, an amino acid residue 13 which is P, S, R, F, H or T, preferably P or S, and an amino acid residue 14 which is S, R, G or T, preferably, S or R.

Furthermore, peptides are also provided which further comprise an N-terminal-flanking amino acid sequence of the formula 1′, 2′-1′, 3′-2′-1′ or 4′-3′-2′-1′ (SEQ ID NO:14) in which each number represents any amino acid residue except cysteine, such that 1′ is bound to R by a peptide bond. In such a case, specific embodiments are provided in which the amino acid residue 1′ is T, P, S, N, F, W, K, H, Q or G,

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preferably T or P, wherein the amino acid residue 2' is S, T, G, P, R, Q, L, A or H, preferably S or T, wherein the amino acid residue 3' is R, S, P, G, A, V, Y or L, preferably S or T, and wherein the amino acid residue 4' is R, S, V, T, G, L or F, preferably R or S.

In a particular embodiment, a peptide is disclosed having at least thirteen and up to forty-five amino acid residues, including an amino acid sequence of the formula, 3'-2'-1'-R-2-L-P-5-6-P-8-9-10 (SEQ ID NO:15), positioned anywhere along the peptide, in which each number represents an amino acid residue, such that 3', 2', 1', 2, 8, and 10 each represents any amino acid residue except cysteine, 5 and 6 each represents a hydrophobic amino acid residue, and 9 represents a hydrophilic amino acid residue except cysteine, each letter being the standard one-letter symbol for the corresponding amino acid, said peptide exhibiting a binding affinity for the SH3 domain of Src. Preferred 13-mers include, but are not limited to, those having an amino acid residue 5 which is a P or M, an amino acid residue 1' which is T, P, S or N, an amino acid residue 2' which is S or T, an amino acid residue 3' which is R or S, and an amino acid residue 10 which is T or R. In all the SH3 domain-binding peptides described herein, the prohibition against the use of the hydrophilic amino acid residue cysteine (C) does not extend beyond the 7-mer "core" sequence and the additional amino acid residues flanking the core up to a total (core + flanking) of about 20 amino acids. That is, the occasional use of a cysteine is not absolutely prohibited. What should be kept in mind is that the potential for the formation of intramolecular disulfide bonds, to form a cyclic structure, be minimized as much as possible. Applicants have found that cyclized structures appear to be disfavored, at least with potential binding peptides of less than about 15 amino acid residues in length. The concern for the formation of cyclized structures comprising the core sequence diminishes with increasing size of the peptide. Presumably, a large

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enough structure, though cyclic, may allow the critical core sequence to adopt a more or less linear conformation.

In particular, specific peptides are disclosed which exhibit binding affinities to SH3 domains. These include the peptides, RSTPRPLPMLPTTR (SEQ ID NO. 62), RSTPRPLPPLPTTR (SEQ ID NO. 67), GILAPPVPPRNTR (SEQ ID NO. 63), VLKRPLPIPPVTR (SEQ ID NO. 64), GPHRRLPPTPATR (SEQ ID NO. 65), and ANPSPATRPLPTR (SEQ ID NO. 66).

Phage clones are also disclosed, along with the amino acid sequences that are responsible for SH3 domain binding. These phage clones are identified in Figure 5.

In other embodiments of the present invention, SH3 domain-binding peptides are contemplated which have a total of 11, 13, 14, 18, 20, 22, 23, 25, 30, 36, 38 or 45 amino acid residues.

The peptides of the present invention, having been disclosed herein, may be prepared by any number of practicable methods, including but not limited to solution-phase synthesis, solid-phase synthesis, protein expression by a transformed host, cleavage from a naturally-derived, synthetic or semi-synthetic polypeptide, or a combination of these techniques.

The SH3 binding peptides exhibit a wide range of biological activity which includes the enhancement (or inhibition, depending on the particular peptide or the nature of the peptide's target molecule, in this case a protein bearing an SH3 domain) of the natural function or biological activity of the peptide's target molecule. For example, the interaction of the binding peptide of the present invention could result in the modulation of the oncogenic activity of the target molecule bearing the SH3 domain. If the target molecule has, in turn, a natural binding partner or ligand, the peptides of the present invention may also exhibit antagonistic or agonistic activity in relation to the biological activity of the natural binding partner.

Thus, it is an object of the present invention to provide a method of activating Src or Src-related protein

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tyrosine kinases by administering an effective amount of the SH3 domain-binding peptides generally described herein. The intensity of the immune response can thus be stimulated, for example, by the increased production of certain lymphokines, such as TNF-alpha and interleukin-1. As is generally known to those of ordinary skill in the art, a more intense immune response may be in order in certain conditions, such as in combating a particularly tenacious infection, viral or otherwise, or a malignancy.

Furthermore, in a specific embodiment of the present invention, a conjugate compound is contemplated which comprises the peptide of the present invention and a second chemical moiety. The second chemical moiety can be selected from a wide variety of chemical compounds including the peptide itself. Typically, however, the second chemical moiety is selected to be other than the peptide of the present invention, including but not limited to an amino acid, a peptide other than an SH3 binding peptide of the present invention, a polypeptide or protein (i.e., the conjugate is a fusion protein), a nucleic acid, a nucleoside, a glycosidic residue (i.e., any sugar or carbohydrate), a label or image-enhancing agent (including metals, isotopes, radioisotopes, chromophores, fluorophores (such as FITC, TRITC, and the like), and enzyme substrates), a drug (including synthetic, semisynthetic, and naturally-occurring compounds), small molecules (e.g., biotin, hormones, factors) and the like.

The peptide of the present invention can be conjugated to the second chemical moiety either directly (e.g., through appropriate functional groups, such as an amine or carboxylic acid group to form, for example, an amine, imine, amide, ester, acyl or other carbon-carbon bond) or indirectly through the intermediacy of a linker group (e.g., an aliphatic or aromatic polyhydroxy, polyamine, polycarboxylic acid, polyolefin or appropriate combinations thereof). Moreover, the term "conjugate," as used herein, is also meant to encompass non-covalent interactions, including but not

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limited to ionic, affinity or other complexation interactions. Preferably, such other non-covalent interactions provide definable, most preferably, isolatable chemical conjugate species.

As described further herein, the peptides of the present invention have been shown to localize within certain cellular compartments which contain Src or Src-related proteins. Consequently, the above-described conjugate can be utilized as a delivery system for introduction of a drug to cells, tissues or organs that include SH3 domain-containing proteins.

It should also be pointed out that the present invention seeks to provide a recombinant construct comprising a nucleic acid or its complement that includes codons or nucleotide sequences encoding a peptide having a region that binds to an SH3 domain, preferably the Src SH3 domain. The recombinant nucleic acid may be a DNA or RNA polynucleotide.

In a specific embodiment, the present invention contemplates a recombinant construct which is a transforming vector. Such vectors include those well known to those of ordinary skill in the art, which effect the transfer or expression of the nucleotide sequence after introduction to a host, such as recombinant plasmid, phage or yeast artificial chromosome. These vectors may be closed circular loops or they may be linearized. The vectors contemplated include those that exist extrachromosomally after host transformation or transfection, as well as those that integrate within or even displace portions of the host chromosome. The vectors may be introduced to the cell with the help of transfection aids or techniques well-known in the art. For example, these aids or techniques may take the form of electroporation, use of calcium chloride, calcium phosphate, DEAE dextran, liposomes or polar lipid reagents known as LIPOFECTIN or LIPOFECTAMINE. In addition, the present invention contemplates the direct introduction of the desired nucleic acid to the host cell, for instance, by injection.

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Transformed host cells are also obtained by the methods of the present invention which are capable of reproducing the polynucleotide sequences of interest and/or expressing the corresponding peptide products. A variety of hosts are contemplated, including prokaryotic and eukaryotic hosts. In particular, bacterial, viral, yeast, animal, and plant cells are potentially transformable hosts. Thus, a method is disclosed to obtain a transformed host cell that can produce, preferably secrete, a peptide having a region that binds to an SH3 domain comprising (a) providing an expression vector, preferably a secretory expression vector, comprising a nucleotide sequence encoding at least one copy of a peptide having a region that binds to an SH3 domain; and (b) introducing the vector to a competent host cell.

The peptides, thus produced, may then be introduced to cells, tissues, organs, or administered to the subject for the purpose of modulating the biochemical activity of the SH3 domain-containing proteins present therein. Accordingly, in specific embodiments of the present invention, compositions are provided which comprise an SH3 domain-binding peptide, including a core sequence and flanking sequences, and a suitable carrier.

The compositions contemplated by the present invention may also include other components, from those that facilitate the introduction or administration of the compositions to those that have their own innate activity, such as a prophylactic, a diagnostic or a therapeutic action. Such innate activity may be distinct from that of the peptides of the present invention or be complementary thereto. In any event, the compositions of the present invention include those that are suitable for administration into mammals, including humans. Preferably, the compositions (including necessarily the carrier) of the present invention are sterile, though others may need only be cosmetically, agriculturally or pharmaceutically acceptable. Still other compositions may be adapted for veterinary use.

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The compositions, including the drug delivery systems described herein, are contemplated to be administered in a variety of ways, such as parenterally, orally, enterally, topically or by inhalation. The compositions may also be adminstered intranasally, opthalmically or intravaginally. Furthermore, the compositions of the invention can take several forms, such as solids, gels, liquids, aerosols or patches.

In another embodiment of the present invention a method is provided of identifying a peptide having a region that binds to an SH3 domain comprising: (a) providing an immobilized target protein comprising an SH3 domain; (b) incubating the immobilized target protein with an aliquot taken from a phage-displayed random peptide library, which library includes peptides having a random sequence of ≥8 amino acid residues; (c) washing unbound phage from the immobilized target protein; (d) recovering the phage bound to the immobilized target protein; and (e) determining the relevant nucleotide sequence of said binding phage nucleic acid and deducing the primary sequence corresponding to the SH3 domain-binding peptide. Preferably, the method further comprises amplifying the titer of the recovered phage and repeating the steps of incubation, washing and recovery to provide SH3 domain-binding peptide-enriched phage.

Any other mode by which the peptide library, random or otherwise, can be "displayed" can be utilized in the present invention, however. Moreover, the present applicants believe that longer *random* peptide sequences (e.g., >6 amino acid residues, preferably >10, and most preferably, >12) provide not only much greater diversity but also a richer degree of secondary structure conducive to binding activity. If the random region of the peptide is less than or equal to an 8-mer, it should preferably not be cyclized.

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5.2. Preparation of Random Peptid Librari s

The preparation and characterization of the preferred phage-displayed random peptide libraries have been described elsewhere. See, for example, Kay, B.K. et al. in Gene (1992) 128:59-65, for a description of the preparation of the phage-displayed random peptide library known as TSAR-9, more below. In particular, by cloning degenerate oligonucleotides of fixed length into bacteriophage vectors, recombinant libraries of random peptides can be generated which are expressed at the amino-terminus of the pIII protein on the surface of M13 viral particles. (There are 3-5 copies of the pIII-fusion on the surface of each particle.) Phage display offers several conveniences: first, the expressed peptides are on the surface of the viral particles and accessible for interactions; second, the recombinant viral particles are stable (i.e., can be frozen, exposed to pH extremes); third, the viruses can be amplified; and fourth, each viral particle contains the DNA encoding the recombinant genome. Consequently, these libraries can be screened by isolating viral particles that bind to targets. The isolates can be grown up overnight, and the displayed peptide sequence responsible for binding can be deduced by DNA sequencing.

These libraries have approximately $>10^8$ different recombinants, and nucleotide sequencing of the inserts suggests that the expressed peptides are indeed random in amino acid sequence. These libraries are referred to herein as TSAR libraries, where TSAR stands for Totally Synthetic Affinity Reagents. The preparation of the TSAR libraries are described further below.

5.3. SH3 Binding Clones And Their Characteristics

Accordingly, peptides have been isolated from an unconstrained random peptide library which exhibit a binding affinity for SH3 domains. Furthermore, the binding affinities exhibited by the disclosed peptides differ in their selectivities with certain peptides showing comparable binding affinities for SH3 domains derived from different

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proteins, while others manifest greater affinities for specific SH3 domains.

The amino acid sequence of various peptides isolated by the present method are listed in Figure 5. As can be seen from this list, certain groups of SH3 domain binding peptides are isolated from three separate random peptide libraries, each based on a different type of random peptide insert, all displayed at the amino-terminus of the pIII protein on the surface of M13 viral particles. Ten clones were isolated from the R8C library, seven from the TSAR-12 library, and seven from the TSAR-9 library. The sequences are presented to highlight the particular amino acid residues believed to bind directly to the SH3 domain, as well as to point out the remaining amino acid resiudes of the random insert and the viral flanking sequences and complementary site amino acid residues common to each group of clones. The frequency with which each particular clone is found in each library is also indicated in Figure 5. Thus, clones T12.SRC3.1 and T12.SRC3.2 are by far the most abundant clones found among the three libraries.

Interestingly, all the binding peptides are found to have the proline-rich amino acid residue motif, which is apparently responsible for binding, the motif being located predominantly at the C-terminal end of the insert, although each clone also contains an insert at the N-terminal end. The significance of this observation is not presently understood, although this finding may indicate the possible importance of the C-terminal viral flanking sequences in SH3 domain binding.

Indeed, a synthetic peptide bearing only the core consensus sequence RPLPPLP (SEQ ID NO:9) was less effective in binding to target SH3 domains than synthetic peptides that also included additional amino acid residues flanking the core sequences. Thus, 13-mers and 14-mers having the sequences RSTPRPLPMLPTTR (SEQ ID NO:62), RSTPRPLPPLPTTR (SEQ ID NO:67), GILAPPVPPRNTR (SEQ ID NO:63), GPHRRLPPTPATR (SEQ ID NO:65), and VLKRPLPIPPVTR (SEQ ID NO:64) have been

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prepared and shown to bind to SH3 domains, such as those of Src and Yes, much more avidly than the 7-mer, RPLPPLP (SEQ ID NO:9). The 13-mer ANPSPATRPLPTR (SEQ ID NO:66) has been shown to have binding affinities comparable to the core consensus sequence. In each case, the 13-mers comprise a 7-mer "core" sequence plus additional amino acid residues flanking same, some of which additional amino acid residues are contributed by the viral flanking sequences.

Thus, in one embodiment of the present invention, a 7-mer core includes a consensus motif of the formula RXLP$\phi\phi$P (SEQ ID NO:71), wherein R is arginine, L is leucine, P is proline, X represents any amino acid except cysteine and ϕ represents a hydrophobic amino acid residue. By "hydrophobic amino acid residue," the applicants mean to include F, Y, W, V, A, I, L, P or M, each letter representing the standard one-letter designation for the corresponding amino acid residue.

Furthermore, a preferred 9-mer peptide comprising two additional amino acids on the C-terminal end of the core sequence is envisioned having a consensus motif of the formula RXLP$\phi\phi$PXψ (SEQ ID NO:10). In this preferred 9-mer consensus motif, the symbol ψ represents a hydrophilic amino acid residue, except cysteine. By "hydrophilic amino acid residue," the applicants mean to include K, R, H, D, E, N, Q, T, S or C, and the other symbols are as defined above. For the purposes of the present invention, a glycine residue (G) may be considered either a hydrophobic or a hydrophilic amino acid residue. The one-letter symbols B and Z, which stand for N or D and Q or E, respectively, are considered hydrophilic amino acid residues.

Particular 13-mer peptides of the present invention include those listed, below. It is noted, however, that not all the following 13-mer peptides correlate strictly to or comply with the preferred 9-mer consensus motif, described above. Those peptides that *do not* comply (indicated in italics, with the non-complying amino acid residues underscored) can, thus, be described as "resembling" those

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that do comply (indicated in normal type) with the preferred 9-mer consensus motif: PGFRELPPLPPSR (SEQ ID NO:72), AQSRPLPIPPETR (SEQ ID NO:73), VLKRPLPIPPVTR (SEQ ID NO:64), *PPNSPLPPLPTHL* (SEQ ID NO:74), *TGRGPLPPLPNDS* (SEQ ID NO:75), *YSTRPVPPITRPS* (SEQ ID NO:76), *SHKSRLPPLPTRP* (SEQ ID NO:77), *YRFRALPSPPSAS* (SEQ ID NO:78), *GPHRRLPPTPATR* (SEQ ID NO:65), *LAQRQLPPTPGRD* (SEQ ID NO:79), *ALQRRLPRTPPPA* (SEQ ID NO:80), *PATRPLPTRPSRT* (SEQ ID NO:81), *YSTRPLPSRPSRT* (SEQ ID NO:82), *XPGRILLLPSEPR* (SEQ ID NO:83), *SGGILAPPVPPRN* (SEQ ID NO:84), RSTRPLPILPRTT (SEQ ID NO:85), STPRPLPMLPTTR (SEQ ID NO:86), STNRPLPMIPTTR (SEQ ID NO:87), *RSTRPLPSLPITT* (SEQ ID NO:88), *STSRPLPSLPTTR* (SEQ ID NO:89), RSTRSLPPLPPTT (SEQ ID NO:90), RSTRQLPIPPTTT (SEQ ID NO:91), STPRPLPLIPTTP (SEQ ID NO:92), *RSTRPLPPTPLTT* (SEQ ID NO:93), and *RSTRPQPPPPITT* (SEQ ID NO:94). Accordingly, other peptides not specifically disclosed, which either comply with or "resemble" the preferred 9-mer consensus motif, can be readily envisioned by those of ordinary skill in the art and are considered to be equivalent to those that are specifically disclosed above. In particular, non-compliance at positions 1 (S, G, and I, in place of R, are tolerated), 3 (V, A, and Q, in place of L, are tolerated), 4 (L, in place of P, is tolerated), 5 (hydrophilic amino acid residues, S, R, and T, are tolerated in place of a hydrophobic amino acid residue), 6 (hydrophilic amino acid residues, R and T, are tolerated in place of a hydrophobic amino acid residue), 7 (T, and S, in place of P, are tolerated), and 9 (P and A are tolerated in place of a hydrophilic amino acid residue) have been observed.

5.3.1. Binding Specificities

It has been discovered that certain of the binding peptides disclosed have a greater relative binding affinity for one SH3 domain over another. Referring now to Figure 8, the relative binding affinities of the various peptides described above toward different SH3 domain targets are graphically presented. As one can see, the relative binding affinities of the respective peptides can differ by orders of

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magnitude. Thus, as shown in Figure 8, the peptide GPHRRLPPTPATR (SEQ ID NO:65), having the relevant sequence of the phage clone identified as T12.SRC3.3, is specific to Src family SH3 domains, including, but not limited to, Src, Yes, Lck, Hck, Fgr, Fyn, and Lyn. This SH3 binding peptide has little affinity for SH3 domains derived from PLCγ or Grb2. On the other hand, the peptide GILAPPVPPRNTR (SEQ ID NO:63), corresponding to the relevant sequence of the phage clone T12.SRC3.1, which is one of the most abundant binding clones found by the present method, binds generically to a broad range of SH3 domains, including Src, PLCγ, and Grb2.

On an intermediate level, the present invention has also uncovered a peptide, VLKRPLPIPPVTR (SEQ ID NO:64), corresponding to the relevant sequence of the phage clone T12.SRC3.6, which is Src preferential; that is, this peptide exhibits strong binding affinities for members of the Src family, some binding affinities for Grb2 proteins, but little binding affinities for PLCγ domains. The peptide ANPSPATRPLPTR (SEQ ID NO:66), corresponding to the relevant sequence of the phage clone T12.SRC3.2, also exhibits Src family specificity similar to GPHRRLPPTPATR (SEQ ID NO:65). The peptides RSTPRPLPMLPTTR (R8C.YES3.5; SEQ ID NO:62) and RSTPRPLPPLPTTR (representative consensus motif; SEQ ID NO:67) are highly specific for SH3 domain of Src, Yes, and other Src-related proteins.

5.4. Further Discussion of Binding Experiments

At the outset it is apparent that the binding affinity of certain peptides to the SH3 domain of Src and Src-related proteins is governed by more than just the presence of the preferred core consensus sequences, RPLPPLP (SEQ ID NO:9) or RPLPMLP (SEQ ID NO:95; i.e., RPLP(P/M)LP, SEQ ID NO:96). Thus, while the synthetic peptides RSTP*RPLPMLP*TTR (R8C.YES3.5; SEQ ID NO:62) and RSTP*RPLPPLP*TTR (consensus; (SEQ ID NO:67) exhibit a strong specific binding affinity for Src SH3, the other synthetic peptides tested also exhibited an avid binding affinity to SH3 domains relative to the 7-

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mer, RPLPPLP (SEQ ID NO:9). These other peptides, GILA*PPVPPR*NTR (SEQ ID NO:63), VLK*RPLPIPP*VTR (SEQ ID NO:64), GPH*RRLPPTP*ATR (SEQ ID NO:65), and ANP*SPATRPL*PTR (SEQ ID NO:66), sport core sequences and flanking sequences that do not closely adhere to the preferred core consensus sequences. Thus, these results suggest that binding affinity to SH3 domains is governed to a large extent by the nature of the amino acid residues flanking the core 7-mer sequence.

The binding characteristics of Src SH3-selected peptides was determined using synthetic biotinylated peptides corresponding to the sequences displayed by Src SH3-selected phage. These biotinylated peptides were assayed for direct binding to immobilized Src SH3-GST. Each of the five library-derived peptides tested were found to bind to Src SH3-GST and Yes SH3-GST over background (Figure 8). Furthermore, a strong correlation was observed between the similarity of a given peptide to the preferred core consensus sequence RPLP(P/M)LP (SEQ ID NO:96) and the peptide's affinity for Src SH3-GST. The core sequence of the clone T12.SRC3.1 (GILA*PPVPPR*NTR; SEQ ID NO:63) appears to provide more generic SH3 domain-binding characteristics.

Experiments comparing the relative binding of various phage clones to SH3 domains taken from a variety of proteins demonstrated the preference of these clones for Src and Src-related SH3 domains over SH3 domains taken from other proteins.

It was further found that while the 7-mer having the consensus sequence RPLPPLP (SEQ ID NO:9) bound to Src SH3-GST only weakly, peptides comprising the consensus sequence flanked by residues encoded by one of the Src SH3-selected clones (R8C.YES3.5), RSTP (SEQ ID NO:97) at the N-terminal end and TTR at the C-terminal end, bound significantly better than any of the peptides tested (Figure 7). Thus, as stated previously, sequences that flank the RPLP(P/M)LP (SEQ ID NO:96) core appear to be important contributors to SH3 binding. It is further surmised that a peptide having or resembling the sequence RSTPAPPVPPRTTR (SEQ ID NO:98) should

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exhibit strong but generic binding to a variety of SH3 domains.

Similarly, it is observed that most of the Src SH3-binding motifs are located near the carboxy-terminus of the random peptides, adjacent to sequences which are fixed in every clone (Figure 5). The exceptional clones tend to possess sequences that resemble motifs that include fixed flanking sequences. This clustering contrasts with previous results, in which binding motifs are distributed throughout the random peptide. Kay, B.K., et al., in Gene (1993) 128:59-65.

The binding of the library-derived Src SH3-binding peptides was compared to that of peptides corresponding to proline-rich regions of natural proteins. Peptides corresponding to SH3-binding regions in human PI-3' Kinase (KISPPTPKPRPPRPLPV; SEQ ID NO:69) and human SOS1.20 (GTVEPVPPPVPPRRRPESA; SEQ ID NO:68), as well as a proline-rich region of the cytoskeletal protein vinculin (LAPPKPPLPEGEV; SEQ ID NO:70), bound Src SH3 much less well than the library-derived peptides (Figure 7).

As mentioned above, the relative specificity of binding was explored. Thus, the relative binding of Src SH3-selected peptides to equal amounts of GST fusions to SH3 domains from different proteins was determined (Figure 8). While all of the library-derived peptides bound the Src and Yes SH3 domains almost equally well, none of the peptides (with the exception of peptide T12.SRC3.1, the most divergent peptide tested) bound the SH3 domains of Grb2, Crk, Abl or PLCγ1 appreciably. Thus, the library-derived peptides, in contrast with a peptide derived from SOS1, exhibit SH3 binding that is relatively specific for Src-family members.

Next, it was determined whether the binding to the Src SH3 domain was qualitatively like the interactions of the SH3 domain and natural proteins found in cell lysates. Thus, radiolabeled proteins were prepared from NIH 3T3 cell lysates and chromatographed over Src SH3-GST immobilized on glutathione linked Sepharose. SDS-PAGE shows that a number

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of proteins can be affinity purified in this manner. The synthesized peptides bind quite well to the Src SH3 domain, as they can compete the binding of radiolabeled proteins from cell lysates to immobilized Src-GST, with an IC_{50} of 1-10 mM (Figure 9). In conclusion, the peptides can efficiently block the interaction of cellular proteins with Src SH3 *in vitro*.

Moreover, *Xenopus laevis* oocytes injected with mRNA encoding constitutively active Src undergo progesterone-induced maturation at an accelerated rate relative to oocytes injected with water or c-Src mRNA. Unger, T.F. and Steele, R.E. in Mol. Cell.Biol. (1992) 12:5485-5498. To explore the ability of the library-derived Src SH3-binding peptides to exert a biochemical effect *in vivo*, the influence of the peptides on the maturation of *Xenopus laevis* oocytes was examined. Hence, stage VI oocytes were injected with peptide, exposed to progesterone, and scored for germinal vesicle breakdown. Figure 10 shows that the rate of maturation was accelerated by approximately one hour when oocytes were injected with the SH3-binding peptide consisting of RPLPPLP (SEQ ID NO:9) flanked by residues from clone T12.SRC3.6 (VLKRPLPIPPVTR; SEQ ID NO:64), but not with water or a peptide corresponding to a proline-rich segment of vinculin (LAPPKPPLPEGEV; SEQ ID NO:70) as controls. The magnitude of this effect is roughly equivalent to that seen with injection of mRNA encoding constituitively active Src. See, e.g., Figure 3B in Unger, T.F. and Steele, R.E., *supra*. This result suggests that the library-derived Src SH3-binding peptide is effectively relieving an inhibitory effect of the Src SH3 domain upon Src PTK activity. This model is consistent with a number of studies which have demonstrated an inhibitory effect of the Src SH3 domain upon Src kinase and transforming activity. See, e.g., Okada, M., et al., *supra*; Murphy, S.M., et al., *supra*; and Superti-Furga, G., et al., *supra*.

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5.5. Diagnostic And Therapeutic Agents Based On SH3 Binding P ptides and Additional Methods of Their Use

As already indicated above, the present invention also seeks to provide diagnostic, prophylactic, and therapeutic agents based on the SH3 binding peptides described herein.

In one embodiment, diagnostic agents are provided, preferably in the form of kits, comprising an SH3 domain-binding peptide and a detectable label conjugated to said peptide directly, indirectly or by complexation, said peptide comprising: (i) a core sequence motif of the formula RXLP$\phi\phi$P (SEQ ID NO:71), wherein X represents any amino acid except cysteine and ϕ represents a hydrophobic amino acid residue, including F, Y, W, V, A, I, L, P, M or G, each letter representing the standard one-letter designation for the corresponding amino acid residue; and (ii) two or more additional amino acid residues flanking said core sequence at its C-terminal end, N-terminal end or both.

The diagnostic agents of the present invention can be used to detect the presence of SH3 domains of a generic or specific type in cells, tissues or organs either *in vitro* or *in vivo*. For *in vivo* applications, the diagnostic agent is preferably mixed with a pharmaceutically acceptable carrier for administration, either enterally, parenterally or by some other route dictated by the needs of the particular application.

In a particular embodiment, for example, an assay based on a fusion product is contemplated which comprises a Src SH3 domain-binding peptide of the invention and a substrate for deregulated or "activated" Src. For instance, a muscle biopsy, taken from a subject suspected of being infected by the Rous sarcoma virus, can be treated with an effective amount of the fusion product. By subsequent analysis of the degree of conversion of the substrate, one can potentially detect infection by the Rous sarcoma virus in the subject, particularly mammals, especially chickens. The presence of the retrovirus, which causes the expression of deregulated or

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"activated" Src, may thus be indicated by unusually high levels of Src as revealed by large amounts of the converted substrate. See, for example, Paxton, W.G. et al., in Biochem. Biophys. Res. Commun. (1994) 200(1):260-267 (detection of phosphorylated tyrosine and serine residues of angiotensin II AT1 receptor, a substrate of Src family tyrosine kinases); another suitable substrate may be the protein p68 (Fumagalli, S. et al., in Nature (1994) 368(6474):871-874; Taylor, S.J. and Shalloway, D., in Ibid. at 867-871.

Alternatively, the enzyme can be isolated by selective binding to a form of the SH3 domain-binding peptides of the present invention (e.g., biotin-peptide conjugate). After isolation of the protein-peptide conjugate complex (e.g., on a column comprising streptavidin), the activity of the enzyme can then be assayed by conventional methods to determine its level of protein kinase activity which can be taken as an indication of the presence of the deregulated or "activated" form of the enzyme. An assay for Src kinase has been described by Klinz and Maness, in Neuroprotocols (a companion to Neuroscience)(1992) 1(3):224-231.

Moreover, the diagnostic agents of the invention can also serve as imaging agents of cells, tissues or organs, especially those that contain proteins with an SH3 domain. For example, neural cells (e.g., neurons, other areas of the brain), osteoclasts, osteoblasts, platelets, immune cells, and other dividing cells are known to express or contain proteins with SH3 domains. Thus, an image can be taken of portions of the body to serve as a baseline for subsequent images to detect physiologic or biochemical changes in the subject's body. For instance, changes in the condition of cellular levels of Src or a transformation of the cellular Src to an "activated" form may be detected using the diagnostic or imaging agents of the present invention.

Accordingly, it has been demonstrated that an SH3-binding peptide tagged with a fluorescence emitter can provide an image of the cytoskeleton. The images are

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presented in Figure 11. As can be seen from Figure 11, panels A, B, and C show the fluorescence image that is obtained on treating NIH 3T3 fibroblasts with SH3 domain-binding peptides modified to include a fluorescent tag. In sharp contrast, panel D shows only a dark image that is produced when the cells are treated with a proline-rich segment of vinculin as a control.

In another embodiment, an SH3 domain-binding peptide-horseradish immunoperoxidase complex or related immunohistochemical agent could be used to detect and quantitate specific receptor molecules in tissues, serum or body fluids. In particular, the present invention provides useful diagnostic reagents for use in immunoassays, Southern or Northern hybridization, and *in situ* assays. Accordingly, the diagnostic agents described herein may be suitable for use *in vitro* or *in vivo*.

In addition, the diagnostic or imaging agent of the present invention is not limited by the nature of the detectable label. Hence, the diagnostic agent may contain one or more such labels including, but not limited to, radioisotope, fluorescent tags, paramagnetic substances, heavy metals, or other image-enhancing agents. Those of ordinary skill in the art would be familiar with the range of label and methods to incorporate or conjugate them into the SH3 domain-binding peptide to form diagnostic agents.

In yet a further embodiment, pharmaceutical compositions are provided comprising an SH3 domain-binding peptide and a pharmaceutically acceptable carrier. In a specific embodiment of the invention, the pharmaceutical composition is useful for the modulation of the activity of SH3 domain-containing proteins. By "modulation" is meant either inhibition or enhancement of the activity of the protein target. Accordingly, a pharmaceutical composition is disclosed comprising an SH3 domain-binding peptide and a pharmaceutically acceptable carrier, said peptide comprising: (i) a 9-mer sequence motif of the formula RXLP$\phi\phi$PXψ (SEQ ID NO:10), wherein X represents any amino acid except cysteine,

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ϕ represents a hydrophobic amino acid residue, and wherein ψ is a hydrophilic amino acid residue except cysteine, each letter representing the standard one-letter designation for the corresponding amino acid residue; and, optionally, (ii) additional amino acid residues flanking the 9-mer sequence at its C-terminal end, N-terminal end or both, up to a total of 45 amino acid residues, including said 9-mer sequence. Preferably, the peptide comprises at least one, more preferably at least two, and most preferably at least three additional amino acids flanking the 9-mer sequence.

As stated above, the therapeutic or diagnostic agents of the invention may also contain appropriate pharmaceutically acceptable carriers, diluents and adjuvants. Such pharmaceutical carriers can be sterile liquids, such as water and oils including those of petroleum, animal, vegetable or synthetic origin, such as peanut oil, soybean oil, mineral oil, sesame oil and the like. Water is a preferred carrier when the pharmaceutical composition is administered intravenously. Saline solutions and aqueous dextrose and glycerol solutions can also be employed as liquid carriers, particularly for injectable solutions. Suitable pharmaceutical excipients include starch, glucose, lactose, sucrose, gelatin, malt, rice, flour, chalk, silica gel, magnesium carbonate, magnesium stearate, sodium stearate, glycerol monostearate, talc, sodium chloride, dried skim milk, glycerol, propylene, glycol, water, ethanol and the like. These compositions can take the form of solutions, suspensions, tablets, pills, capsules, powders, sustained-release formulations and the like. Suitable pharmaceutical carriers are described in "Remington's Pharmaceutical Sciences" by E.W. Martin.

Such compositions will contain an effective therapeutic amount of the active compound together with a suitable amount of carrier so as to provide the form for proper administration to the subject. While intravenous injection is a very effective form of administration, other modes can be employed, including but not limited to intramuscular,

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intraperitoneal, and subcutaneous injection, and oral, nasal, enteral, and parenteral administration.

The therapeutic agents and diagnostic agents of the instant invention are used for the treatment and/or diagnosis of animals, and more preferably, mammals including humans, as well as dogs, cats, horses, cows, pigs, guinea pigs, mice and rats. Accordingly, other methods contemplated in the present invention, include, but are not limited to, a method of modulating, i.e., inhibiting or enhancing, bone resorption in a mammal (see, e.g., Hall, T.J., in Biochem. Biophys. Res. Commun. (1994) 199(3):1237-44), a method of disrupting protein tyrosine kinase-mediated signal transduction pathways or a method of regulating the processing, trafficking or translation of RNA in a cell by introducing or administering an effective amount of an SH3 domain-binding peptide of the present invention (see, e.g., Taylor, S.J. and Shalloway, D., *supra*).

The diagnostic or therapeutic agents of the present invention can be modified by attachment to soluble macromolecules such as proteins, polysaccharides, or synthetic polymers. For example, the peptide could be coupled to styrene-maleic acid copolymers (see, e.g., Matsumura and Maeda, Cancer Res. (1986) 46:6387), methacrylamide copolymers (Kopececk and Duncan, J. Controlled Release (1987) 6:315), or polyethylene glycol (PEG) (e.g., Hershfield and Buckley, N. Engl. J. Med. (1987) 316:589; Ho et al., Drug Metab. Dispos. (1986) 14:349; Chua et al., Ann. Intern. Med. (1988) 109:114). The agents, if desired, are further targeted by attachment to an antibody, especially a monoclonal antibody. Such antibodies include but are not limited to chimeric, single chain, Fab fragments, and Fab expression libraries. In one embodiment the agent is coupled to the macromolecule via a degradable linkage so that it will be released *in vivo* in its active form.

In another embodiment, the therapeutic or diagnostic agent may be delivered in a vesicle, in particular a liposome. See, Langer, Science (1990) 249:1527-1533; Treat

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et al., in Liposomes in the Therapy of Infectious Disease and Cancer, Lopez-Berestein and Fidler (eds.), Liss, New York (1989) pp. 353-365; Lopez-Berestein, ibid., pp. 317-327.

In yet another embodiment, the therapeutic or *in vivo* diagnostic agent can be delivered in a controlled release system. In one embodiment, a pump may be used (see Langer, *supra*; Sefton, CRC Crit. Ref. Biomed. Eng. (1987) 14:201; Buchwald et al., Surgery (1980) 88:507; Saudek et al., N. Engl. J. Med. (1989) 321:574). In another embodiment, polymeric materials may be used (see Medical Applications of Controlled Release, Langer and Wise (eds.), CRC Pres., Boca Raton, Florida, 1974; Controlled Drug Bioavailability, Drug Product Design and Performance, Smolen and Ball (eds.) Wiley, New York 1984; Raner and Peppas, J. Macromol. Sci. Rev. Macromol. Chem. (1983) 23:61; see, also, Levy et al., Science (1985) 228:190; During et al., Ann. Neurol. (1989) 25:351; Howard et al., J. Neurosurg. (1989) 71:105). In a preferred embodiment, a controlled release system may be placed next to the therapeutic target, thus requiring only a fraction of the systemic dose (see, e.g., Goodson, in Medical Applications of Controlled Release, *supra*, (1984) 2:115-138). It will be recognized by one of ordinary skill in the art that a particular advantage of the invention is that a peptide will not be subject to the problems of denaturation and aggregation associated with proteins held in the warm, most environment of a body in a controlled release system.

Other controlled release systems are discussed in the review by Langer, in Science (1990) 249:1527-1533.

5.6. Identification of Compounds that Affect Binding of SH3 Domain-containing Proteins and their Ligands

A common problem in the development of new drugs is that of identifying a single, or a small number, of compounds that possess a desirable characteristic from among a background of a large number of compounds that lack that desired characteristic. This problem arises both in the testing of

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compounds that are natural products from plant, animal, or microbial sources and in the testing of man-made compounds. Typically, hundreds, or even thousands, of compounds are randomly screened by the use of *in vitro* assays such as those that monitor the compound's effect on some enzymatic activity or its ability to bind to a reference substance such as a receptor or other protein.

The compounds which pass this original screening test are known as "lead" compounds. These lead compounds are then put through further testing, including, eventually, *in vivo* testing in animals and humans, from which the promise shown by the lead compounds in the original *in vitro* tests is either confirmed or refuted. See Remington's Pharmaceutical Sciences, 1990, A.R. Gennaro, ed., Chapter 8, pages 60-62, Mack Publishing Co., Easton, PA; Ecker and Crooke, 1995, Bio/Technology 13:351-360.

There is, of course, a continual need for new compounds to be tested in the *in vitro* assays that make up the first testing step described above. There is also a continual need for new assays by which the pharmacological activities of these compounds may be tested. It is an object of the present invention to provide such new assays to determine whether a candidate compound is capable of affecting the binding between a protein or polypeptide containing an SH3 domain and a ligand of the SH3 domain. A compound capable of affecting this binding would be useful as a means of modulating the pharmacological activity of proteins or polypeptides containing the SH3 domain. The present invention provides suitable ligands for SH3 domains for use in such assays. Such assays can be performed where the SH3 domains include, but are not limited to, SH3 domains from Cortactin, Nck, Abl, PLCγ, Src, p53bp2, Crk, Yes, and Grb2.

The present invention provides methods of identifying a compound that affects the binding of a molecule comprising an SH3 domain and a ligand of the SH3 domain. The effect on binding can be an increase or decrease in total amount of

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binding or in affinity of bidning. Preferably, the effect is an inhibition (reduction in or loss of binding).

Accordingly, the invention provides a method of identifying an inhibitor of the binding between a first molecule comprising an SH3 domain and a second molecule that binds to the SH3 domain comprising incubating one or more compounds from which it is desired to select such an inhibitor with the first molecule and the second molecule under conditions conducive to binding and detecting the one or more compounds that inhibit binding of the first molecule to the second molecule.

In a particular embodiment of the above-described metnod, the second molecule is obtained by:

(i) screening a peptide library with the SH3 domain to obtain peptides that bind the SH3 domain;

(ii) determining a consensus sequence for the peptides obtained in step (i);

(iii) producing a peptide comprising the consensus sequence;

wherein the second molecule comprises the peptide comprising the consensus sequence.

In another embodiment, the second molecule is obtained by:

(i) screening a peptide library with the SH3 domain to obtain peptides that bind the SH3 domain;

(ii) determining a consensus sequence for the peptides obtained in step (i);

(iii) searching a database to identify amino acid sequences that resemble the consensus sequence of step (ii);

(iv) producing a peptide comprising an amino acid sequence identified in step (iii);

wherein the second molecule comprises the peptide comprising an amino acid sequence identified in step (iii).

Second molecules that bind SH3 domains can be obtained by, *e.g.*, the use of diversity libraries, such as random or combinatorial peptide or nonpeptide libraries which can be screened for molecules that specifically bind to SH3 domains.

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Many libraries are known in the art that can be used, *e.g.*, chemically synthesized libraries, recombinant (*e.g.*, phage display libraries), and *in vitro* translation-based libraries.

Examples of chemically synthesized libraries are described in Fodor et al., 1991, Science 251:767-773; Houghten et al., 1991, Nature 354:84-86; Lam et al., 1991, Nature 354:82-84; Medynski, 1994, Bio/Technology 12:709-710; Gallop et al., 1994, J. Medicinal Chemistry 37(9):1233-1251; Ohlmeyer et al., 1993, Proc. Natl. Acad. Sci. USA 90:10922-10926; Erb et al., 1994, Proc. Natl. Acad. Sci. USA 91:11422-11426; Houghten et al., 1992, Biotechniques 13:412; Jayawickreme et al., 1994, Proc. Natl. Acad. Sci. USA 91:1614-1618; Salmon et al., 1993, Proc. Natl. Acad. Sci. USA 90:11708-11712; PCT Publication No. WO 93/20242; and Brenner and Lerner, 1992, Proc. Natl. Acad. Sci. USA 89:5381-5383.

Examples of phage display libraries are described in Scott and Smith, 1990, Science 249:386-390; Devlin et al., 1990, Science, 249:404-406; Christian, R.B., et al., 1992, J. Mol. Biol. 227:711-718); Lenstra, 1992, J. Immunol. Meth. 152:149-157; Kay et al., 1993, Gene 128:59-65; and PCT Publication No. WO 94/18318 dated August 18, 1994.

In vitro translation-based libraries include but are not limited to those described in PCT Publication No. WO 91/05058 dated April 18, 1991; and Mattheakis et al., 1994, Proc. Natl. Acad. Sci. USA 91:9022-9026.

By way of examples of nonpeptide libraries, a benzodiazepine library (*see e.g.*, Bunin et al., 1994, Proc. Natl. Acad. Sci. USA 91:4708-4712) can be adapted for use. Peptoid libraries (Simon et al., 1992, Proc. Natl. Acad. Sci. USA 89:9367-9371) can also be used. Another example of a library that can be used, in which the amide functionalities in peptides have been permethylated to generate a chemically transformed combinatorial library, is described by Ostresh et al. (1994, Proc. Natl. Acad. Sci. USA 91:11138-11142).

Screening the libraries can be accomplished by any of a variety of commonly known methods. See, *e.g.*, the following references, which disclose screening of peptide libraries:

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Parmley and Smith, 1989, Adv. Exp. Med. Biol. 251:215-218; Scott and Smith, 1990, Science 249:386-390; Fowlkes et al., 1992; BioTechniques 13:422-427; Oldenburg et al., 1992, Proc. Natl. Acad. Sci. USA 89:5393-5397; Yu et al., 1994, Cell 76:933-945; Staudt et al., 1988, Science 241:577-580; Bock et al., 1992, Nature 355:564-566; Tuerk et al., 1992, Proc. Natl. Acad. Sci. USA 89:6988-6992; Ellington et al., 1992, Nature 355:850-852; U.S. Patent No. 5,096,815, U.S. Patent No. 5,223,409, and U.S. Patent No. 5,198,346, all to Ladner et al.; Rebar and Pabo, 1993, Science 263:671-673; and PCT Publication No. WO 94/18318.

In a specific embodiment, screening can be carried out by contacting the library members with an SH3 domain immobilized on a solid phase and harvesting those library members that bind to the SH3 domain. Examples of such screening methods, termed "panning" techniques are described by way of example in Parmley and Smith, 1988, Gene 73:305-318; Fowlkes et al., 1992, BioTechniques 13:422-427; PCT Publication No. WO 94/18318; and in references cited hereinabove.

In another embodiment, the two-hybrid system for selecting interacting proteins in yeast (Fields and Song, 1989, Nature 340:245-246; Chien et al., 1991, Proc. Natl. Acad. Sci. USA 88:9578-9582) can be used to identify molecules that specifically bind to SH3 domains.

A typical assay of the present invention consists of at least the following components: (1) a molecule (*e.g.*, protein or polypeptide) comprising an SH3 domain; (2) a ligand of the SH3 domain; (3) a candidate compound, suspected of having the capacity to affect the binding between the protein containing the SH3 domain and the ligand. The assay components may further comprise (4) a means of detecting the binding of the protein comprising the SH3 domain and the ligand. Such means can be *e.g.*, a detectable label affixed to the protein, the ligand, or the candidate compound.

In another specific embodiment, the invention provides a method of identifying a compound that affects the binding of

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a molecule comprising an SH3 domain and a ligand of the SH3 domain comprising:

(a) contacting the SH3 domain and the ligand under conditions conducive to binding in the presence of a candidate compound and measuring the amount of binding between the SH3 domain and the ligand;

(b) comparing the amount of binding in step (a) with the amount of binding known or determined to occur between the molecule and the ligand in the absence of the candidate compound, where a difference in the amount of binding between step (a) and the amount of binding known or determined to occur between the molecule and the ligand in the absence of the candidate compound indicates that the candidate compound is a compound that affects the binding of the molecule comprising an SH3 domain and the ligand.

A kit is provided that comprises, in one or more containers, one or more components of the assay of the invention, *e.g.*, a first molecule comprising an SH3 domain and a second molecule that binds to the SH3 domain.

In one embodiment, the assay comprises allowing the protein or polypeptide containing an SH3 domain to contact the ligand of the SH3 domain in the presence and in the absence of the candidate compound under conditions such that binding of the ligand to the protein containing an SH3 domain will occur unless that binding is disrupted or prevented by the candidate compound. By detecting the amount of binding of the ligand to the protein containing an SH3 domain in the presence of the candidate compound and comparing that amount of binding to the amount of binding of the ligand to the protein or polypeptide containing an SH3 domain in the absence of the candidate compound, it is possible to determine whether the candidate compound affects the binding and thus is a useful lead compound for the modulation of the activity of proteins containing the SH3 domain. The effect of the candidate compound may be to either increase or decrease the binding.

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One version of an assay suitable for use in the present invention comprises binding the protein containing an SH3 domain to a solid support such as the wells of a microtiter plate. The wells contain a suitable buffer and other substances to ensure that conditions in the wells permit the binding of the protein or polypeptide containing an SH3 domain to its ligand. The ligand and a candidate compound are then added to the wells. The ligand is preferably labeled, *e.g.*, it might be biotinylated or labeled with a radioactive moiety, or it might be linked to an enzyme, *e.g.*, alkaline phosphatase. After a suitable period of incubation, the wells are washed to remove any unbound ligand and compound. If the candidate compound does not interfere with the binding of the protein or polypeptide containing an SH3 domain to the labeled ligand, the labeled ligand will bind to the protein or polypeptide containing an SH3 domain in the well. This binding can then be detected. If the candidate compound interferes with the binding of the protein or polypeptide containing an SH3 domain and the labeled ligand, label will not be present in the wells, or will be present to a lesser degree than is the case when compared to control wells that contain the protein or polypeptide containing an SH3 domain and the labeled ligand but to which no candidate compound is added. Of course, it is possible that the presence of the candidate compound will increase the binding between the protein or polypeptide containing an SH3 domain and the labeled ligand. Alternatively, the ligand can be affixed to solid substrate during the assay.

The present invention provides ligands capable of binding SH3 domains that are suitable for incorporation into assays such as those described above. Ligands provided by the present invention include those SH3 domain-binding amino acid sequences disclosed in Tables 1-13 below and proteins or polypeptides containing those amino acid sequences. Also provided are nucleic acids encoding the SH3 domain-binding amino acid sequences disclosed in Tables 1-13 below.

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6. EXAMPLES

6.1. Preparation of the TSAR-9 Library

6.1.1. Synth sis and Assembly of Oligonucl otides

Figure 1 shows the formula of the oligonucleotides and the assembly scheme used in construction of the TSAR-9 library. The oligonucleotides were synthesized with an applied Biosystems 380a synthesizer (Foster City, CA), and the full-length oligonucleotides were purified by HPLC.

Five micrograms of each of the pair of oligonucleotides were mixed together in buffer (10 mM Tris-HCl, pH 8.3, 15 mM KCl, 0.001% gelatin, 1.5 mM magnesium chloride), with 0.1 % Triton X-100, 2 mM dNTP's, and 20 units of Taq DNA polymerase. The assembly reaction mixtures were incubated at 72 °C for 30 seconds and then 30 °C for 30 seconds; this cycle was repeated 60 times. It should be noted that the assembly reaction is not PCR, since a denaturation step was not used. Fill-in reactions were carried out in a thermal cycling, device (Ericomp, LaJolla, CA) with the following protocol: 30 seconds at 72 °C, 30 seconds at 30 °C, repeated for 60 cycles. The lower temperature allows for annealing of the six base complementary region between the two sets of the oligonucleotide pairs. The reaction products were phenol/chloroform extracted and ethanol precipitated. Greater than 90% of the nucleotides were found to have been converted to double stranded synthetic oligonucleotides.

After resuspension in 300 μL of buffer containing 10 mM Tris-HCI, pH 7.5, 1 mM EDTA (TE buffer), the ends of the oligonucleotide fragments were cleaved with *Xba* I and *Xho* I (New England BioLabs, Beverly, MA) according to the supplier's recommendations. The fragments were purified by 4% agarose gel electrophoresis. The band of correct size was removed and electroeluted, concentrated by ethanol precipitation and resuspended in 100 μL TE buffer. Approximately 5% of the assembled oligonucleotides can be expected to have internal *Xho* I or *Xba* I sites; however, only the full-length molecules were used in the ligation step of

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the assembly scheme. The concentration of the synthetic oligonucleotide fragments was estimated by comparing the intensity on an ethidium bromide stained gel run along with appropriate quantitated markers. All DNA manipulations not described in detail were performed according to Maniatis, *supra*.

To demonstrate that the assembled enzyme digested oligonucleotides could be ligated, the synthesized DNA fragments were examined for their ability to self-ligate. The digested fragments were incubated overnight at 18 °C in ligation buffer with T4 DNA ligase. When the ligation products were examined by agarose gel electrophoresis, a concatamer of bands was visible upon ethidium bromide staining. As many as five different unit length concatamer bands (i.e., dimer, trimer, tetramer, pentamer, hexamer) were evident, suggesting that the synthesized DNA fragments were efficient substrates for ligation.

6.1.2. Construction of Vectors

The construction of the M13 derived phage vectors useful for expressing a TSAR library has been recently described (Fowlkes, D. et al. BioTech. (1992) 13:422-427). To express the TSAR-9 library, an M13 derived vector, m663, was constructed as described in Fowlkes. The m663 vector contains the pIII gene having a *c-myc*-epitope, i.e., as a stuffer fragment, introduced at the mature N-terminal end, flanked by *Xho* I and *Xba* I restriction sites (see also, Figure I of Fowlkes).

6.1.3. Expression of the TSAR-9 Library

The synthesized oligonucleotides were then ligated to *Xho* I and *Xba* I double-digested m663 RF DNA containing, the pIII gene (Fowlkes) by incubation with ligase overnight at 12 °C. More particularly, 50 ng of vector DNA and 5 ng of the digested synthesized DNA and was mixed together in 50 μL ligation buffer (50 mM Tris, pH 8.0, 10 mM $MgCl_2$, 20 mM DTT, 0.1 mM ATP) with T4 DNA ligase. After overnight ligation at

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12°C, the DNA was concentrated by ethanol precipitation and washed with 70% ethanol. The ligated DNA was then introduced into *E. coli* (DH5αF'; GIBCO BRL, Gaithersburg, MD) by electroporation.

A small aliquot of the electroporated cells was plated and the number of plaques counted to determine that 10^8 recombinants were generated. The library of *E. coli* cells containing recombinant vectors was plated at a high density (~400,000 per 150 mM petri plate) for a single amplification of the recombinant phage. After 8 hr, the recombinant bacteriophage were recovered by washing each plate for 18 hr with SMG buffer (100 mM NaCl, 10 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 0.05% gelatin) and after the addition of glycerol to 50% were frozen at -80 °C. The TSAR-9 library thus formed had a working titer of ~2 x 10^{11} pfu/ml.

6.2. Preparation of the TSAR-12 Library

Figure 2 shows the formula for the synthetic oligonucleotides and the assembly scheme used in the construction of the TSAR-12 library. As shown in Figure 2, the TSAR-12 library was prepared substantially the same as the TSAR-9 library described in Section 6.1 above with the following exceptions: (1) each of the variant non-predicted oligonucleotide sequences, i.e., NNB, was 30 nucleotides in length, rather than 54 nucleotides; (2) the restriction sites included at the 5' termini of the variant, non-predicted sequences were *Sal* I and *Spe* 1, rather than *Xho* I and *Xba* I; and (3) the invariant sequence at the 3' termini to aid annealing of the two strands was GCGGTG and CGCCAC rather than CCAGGT and GGTCCA (5' to 3').

After synthesis including numerous rounds of annealing and chain extension in the presence of dNTP's and Taq DNA polymerase, and purification as described above in Section 6.1.1, the synthetic double stranded, oligonucleotide fragments were digested with *Sal* I and *Spe* I restriction enzymes and ligated with T4 DNA ligase to the nucleotide sequence encoding the M13 pIII gene contained in the m663

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vector to yield a library of TSAR-expression vectors as described in Sections 6.1.2 and 6.1.3. The ligated DNA was then introduced into *E. coli* (DH5αF'; GIBCO BRL, Gaithersburg, MD by electroporation. The library of *E. coli* cells were plated at high density (~400,000 per 150 mm petri plate) for amplification of the recombinant phage. After about 8 hr, the recombinant bacteriophage were recovered by washing, for 18 hr with SMG buffer and after the addition of glycerol to 50% were frozen at -80 °C.

The TSAR-12 library thus formed had a working titer of ~2 x 10^{11} pfu/mL.

6.3. Characterization of the TSAR-9 and -12 Libraries

The inserted synthetic oligonucleotides for each of the TSAR libraries, described in Sections 6.1 and 6.2 above, had a potential coding complexity of 20^{36} (~10^{47}) and 20^{20}, respectively, and since ~10^{14} molecules were used in each transformation experiment, each member of these TSAR libraries should be unique. After plate amplification the library solution or stock has 10^{4} copies of each member/mL.

It was observed that very few (<10%) of the inserted oligonucleotide sequences characterized so far in both of the libraries have exhibited deletions or insertions. This is likely a reflection of the accuracy assembling the oligonucleotides under the conditions used and the fact that certain types of mutations (i.e., frame-shifts) would not be tolerated as pIII an essential protein for phage propagation.

In order to determine whether any coding bias existed in the variant non-predicted peptides expressed by these libraries, perhaps due to biases imposed *in vitro* during synthesis of the oligonucleotides or *in vivo* during expression by the reproducing phage, inserts were sequenced as set forth below.

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6.3.1. Characterization of TSAR-9 Library

Inserted synthetic oligonucleotide fragments of 23 randomly chosen isolates were examined from the TSAR-9 library. Individual plaques were used to inoculate I ml of 2XYT broth containing *E. coli* (DH5αF') cells and the cultures were allowed to grow overnight at 37°C with aeration. DNA was isolated from the culture supernatants according to Maniatis, *supra*. Twenty-three individual isolates were sequenced according to the method of Sanger (Proc. Natl. Acad. Sci. USA (1979) 74:5463-5467) using as a primer the oligonucleotide 5'-AGCGTAACGATCTCCCG (SEQ ID NO. 99), which is 89 nucleotides downstream of the pIII gene cloning site of the m663 vector used to express the TSARS.

Nucleotide sequences and their encoded amino acid sequences were analyzed with the MacVector computer program (IBI, New Haven, CT). The Microsoft EXCEL program was used to evaluate amino acid frequencies. Such analyses showed that the nucleotide codons coding for and hence most amino acids, occurred at the expected frequency in the TSAR-9 library of expressed proteins. The notable exceptions were glutamine and tryptophan, which were over- and under-represented, respectively.

It is of interest to note the paucity of TAG stop codons in the inserts, i.e., only 2 of ~200 isolates characterized contained a TAG stop codon. About half $[1-(47/48)^{36}]$ of the phage inserts were expected to have at least one TAG codon in view of the assembly scheme used. However, most of the TAG-bearing phage appear to have been lost from the library, even though the bacterial host was *supE*. This may be a consequence of suppression being less than 100% effective.

The amino acids encoded by the inserted double stranded synthesized oligonucleotide sequences, excluding the fixed PG-encoding centers, were concatenated into a single sequence and the usage frequency determined for each amino acid using the Microsoft EXCEL program. These frequencies were compared to that expected from the assembly scheme of the oligonucleotides, and the divergence from expected values

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represented by the size of the bars above and below the baseline. Chi square analysis was used to determine the significance of the deviations. The majority of amino acids were found to occur at the expected frequency, with the notable exceptions that glutamine and tryptophan were somewhat over- and under-represented, respectively. Thus, except for the invariant Pro-Gly, any position could have any amino acid; hence, the sequences are unpredicted or random.

6.3.2. Characterization of TSAR-12 Library

Approximately 10 randomly chosen inserted oligonucleotides from the TSAR-12 library were examined by DNA sequencing as described above in Section 6.3.1. The isolates were chosen at random from the TSAR-12 library and prepared for sequencing, as were the TSAR-9 isolates. Analysis showed that except for the invariant Gly any position could have any amino acid; hence, the sequences are unpredicted or random.

6.4. Preparation of R8C Library

Referring now to Figure 3, two oligonucleotides were synthesized on an Applied Biosystems Model 380a machine with the sequence 5′-TGACGTCTCGAGTTGTNNKNNKNNKNNKNNKNNKNNKNNKTGTGGATCTAGAAGGATC-3′ (SEQ ID NO:31) and 5′-GATCCTTCTAGATCC-3′ (SEQ ID NO:32), where N is an equimolar ratio of deoxynucleotides A, C, G, and T, and K is an equimolar ratio of G and T. Fifty pmol of each oligonucleotide was incubated at 42 °C for 5 min, then 37 °C for 15 min, in 50 μL of Sequenase™ buffer (U.S. Biochemicals, Cleveland, OH) with 0.1 μg/μL acetylated BSA, and 10 mM DTT. After annealing, 10 units of Sequenase™ (U.S. Biochemicals) and 0.2 mM of each dNTP were added and incubated at 37 °C for 15 min. The sample was then heated at 65 °C for 2 hr, digested with 100 units of both *Xho* I and *Xba* I (New England BioLabs, Beverly, MA), phenol extracted, ethanol precipitated, and resolved on a 15% non-denaturing polyacrylamide gel. The assembled, digested fragment was gel

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purified prior to ligation. The vector, m663 (Fowlkes, D. et al. Biotech. (1992) 13:422-427), was prepared by digestion with *Xho* I and *Xba* I, calf alkaline phosphatase (Boehringer Mannheim, Indianapolis, IN) treatment, phenol extracted, and purified by agarose gel electrophoresis. To ligate, 20 μg vector was combined with 0.2 μg insert in 3 mL with T4 DNA ligase (Boehringer Mannheim), according to the manufacturer. After removal of the protein and buffer by phenol extraction and ethanol precipitation, the ligated DNA was electroporated into XL1-Blue *E. coli* (Stratagene, San Diego, CA) and plated for eight hours at 37 °C. To recover the recombinant phage, the top agar was collected with a spatula, mixed with an equal volume of 100 mM NaCl, 10 mM $MgCl_2$, and 50 mM Tris-HCl (pH7.5), and disrupted by two passes through an 18-gauge syringe needle. The bacterial cells were removed by centrifugation, and phage particles were collected by polyethylene glycol precipitation and stored at -70 °C in 25% glycerol. The library had 10^6 total recombinants and a working titer of 6×10^{12} pfu/mL.

Members of the library were checked for inserts by the polymerase chain reaction (Saiki, et al. Science (1988) 239:487-491). Individual plaques on a petri plate were touched with a sterile toothpick and the tip was stirred into 2xYT with F' *E. coli* bacteria and incubated overnight at 37 °C with aeration. Five microliters of the phage supernatant were then transferred to new tubes containing buffer (67 mM Tris-HCl, pH 8.8/10 mM β- mercaptoethanol/16.6 mM ammonium sulfate/6.7 mM EDTA/50 μg bovine serum albumin per mL), 0.1 mM deoxynucleotide triphosphates, and 1.25 units of *Taq* DNA polymerase (Boehringer Mannheim, Indianapolis, IN) with 100 pmoles of oligonucleotide primers. The primers flanked the cloning site in gene III of m663 (5'-TTCACCTCGAAAGCAAGCTG-3' (SEQ ID NO:100) and 5'-CCTCATAGTTAGCGTAACG-3' (SEQ ID NO:101)). The assembly reactions were incubated at 94 °C for 1 min, 56 °C for 2 min, and 72 °C for 3 min; this cycle was repeated 24 times. The reaction products were then resolved by electrophoresis on a NuSieve 2.0% agarose gel (FMC,

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Rockland, ME). Gels revealed that for 20 plaques tested, all were recombinant and had single inserts of the expected size.

Based on the sample size of the library, it was anticipated that 100% of the recombinants had single inserts. However, all of the SH3-binding phage isolated from the R8C library had double-inserts. Such phage are presumed rare (i.e., <5%) within the library, yet because the SH3-binding peptide appears to need to be linear they were selected for by our screening methods. Most likely they were formed during the generation of the library; one scenario is that the inserts ligated together to form head-to-head dimers and that they were subsequently cloned into m663 DNA by ligation with the vector's *Xho I* sticky end and by illegitimate ligation with the vector's *Xba I* site (see, Figure 4).

6.5. Preparation Of Target-Coated Microtiter Wells

6.5.1. Preparation Of GST-SH3 Fusion Proteins

The preparation of Src-GST fusion protein was first described by Smith and Johnson, in Gene (1988) 67:31, the disclosure of which is incorporated by reference herein. Briefly, pGEX-derived (Pharmacia, Piscataway, NJ) constructs expressing GST fusion proteins containing the SH3 domains of Src, Grb2, Crk, Abl, or PLCγ were obtained from Dr. Channing Der (University of North Carolina at Chapel Hill); a construct expressing the SH3 domain of Yes was obtained from Dr. Marius Sudol (Rockefeller University). The use of the pGEX bacterial expression vector for the production of GST-SH3 fusion proteins is well-known to those in the art. See, e.g., Cicchetti, P. et al., in Science (1992) 257:803-806. Briefly, the coding region for a particular SH3 domain can be fused in-frame at the Bam HI site of pGEX-2T. Thus, fusion proteins were prepared as per the manufacturer's instructions, and quantified by Coomassie Blue staining of SDS-polyacrylamide gels. Microtiter wells were coated with 5-20 μg GST-SH3 fusion protein in 100 mM $NaHCO_3$, pH 8.5, blocked with 100 mM $NaHCO_3$ (pH 8.5) 1% BSA, and washed. All

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washes consisted of five applications of 1XPBS, 0.1% Tween 20, 0.1% BSA (Buffer A). Where appropriate, the amount of protein bound to each well was quantified with an anti-GST antibody-based ELISA (Pharmacia, Piscataway, NJ), and with a GST-binding phage, isolated during the course of this work.

6.5.2. Coating of Microtiter Wells

Bacterially expressed Src SH3 glutathione-S-transferase (Src-GST) fusion protein was purified from bacterial lysates using glutathione agarose 4B (Pharmacia), according to the manufacturer's instructions. Bound Src-GST fusion protein was eluted from the glutathione agarose with 10 mM glutathione in PBS. Microtiter wells were then coated with Src-GST fusion protein (1-10 μg/well, in 50 mM $NaHCO_3$, pH 8.5) overnight at 4 °C. To block non-specific binding of phage, 100 μL 1% BSA in 100 mM $NaHCO_3$, pH 8.5, was added to each well and allowed to incubate at room temperature for 1 hour. The wells were then washed five times with 200 μL PBS, 0.1% Tween 20, 0.1% BSA (Buffer A).

6.6. Biopanning And Subsequent Characterization Of Phage-Displayed Random Peptide Libraries With Src-GST Fusion Protein As Target Molecule

6.6.1. Isolation of Src SH3-Binding Phage

Library screens were performed as previously described. Kay, B.K., et al., in Gene (1993) 128:59-65. Briefly, 1 X 10^{11} pfu TSAR 9, TSAR 12, or R8C phage in Buffer A were incubated in a Src SH3-GST-coated well for 2 hours. The wells were washed, and bound phage were eluted with 100 μL 50 mM glycine·HCl (pH 2.2), transferred to a new well, and neutralized with 100 mL 200 mM $NaHPO_4$ (pH 7.0). Recovered phage were used to infect 1 x 10^9 DH5αF′ *E. coli* cells in 20 mL 2xYT; the infected cells were grown overnight, resulting in a 1000- to 10,000-fold amplification of phage titer. Amplified phage were panned twice more, as above, excepting the amplification step. Binding phage recovered after the third round of panning were plated at a low density on a lawn

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of DH5αF′ *E. coli* cells to yield isolated plaques for clonal analysis. Isolated plaques were used to produce small cultures from which phage stocks and DNA were recovered for phage binding experiments and dideoxy sequencing (Sanger, F., et al., in Proc. Natl. Acad. Sci. USA (1977) 74:5463-5467), respectively. Clones were confirmed as binding the SH3 domain by applying equal titers of phage to wells containing Src SH3-GST or GST alone, and titering the number of eluted particles from each well, or detecting bound phage with an anti-phage antibody-based ELISA (Pharmacia).

Indeed, the ability of isolated phage clones to bind to several SH3 domains derived from a variety of different proteins can be investigated by the manner described above. GST-SH3 fusion proteins containing SH3 domains from a variety of different proteins are bound to microliter wells. An aliquot of the aforementioned phage stocks (50 μL) is introduced into wells containing the different GST-SH3 fusion proteins. After room temperature incubation for 1-2 hours, the liquid contents of the microtiter plates are removed, and the wells are washed 5 times with 200 μL Buffer A. Bound phage are eluted with 100 μL 50 mM glycine (pH 2.2), transferred to a new well, and neutralized with 100 μL 200 mM $NaHPO_4$ (pH 7.0). The phage are diluted 10^{-3}- to 10^{-6}-fold, and aliquots are plated onto lawns of DH5αF′ *E. coli* cells to establish the number of plaque forming units in the output sample. From these experiments, the relative specificity of different Src SH3 binding clones for SH3 domains derived from other proteins is determined.

6.6.2. Phage ELISA and Nucleotide Sequencing

To evaluate the binding of isolates to various targets proteins, enzyme-linked-immuno-assays (ELISA) were also performed. Bacterial cultures were infected with phage isolates and cultured overnight in 2XYT at 37 °C. The cells were spun down and 25 mL of supernatant was added to microtiter plate wells coated with 50 μL of protein (1 mg/mL

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in 100 mM $NaHCO_3$, pH 8.4; overnight at 4 °C or for a few hours at room temperature) and blocked (1 mg/mL BSA in 100 mM $NaHCO_3$, pH 8.4; for about one hour). The phage are incubated in the well with 25 μL of PBS-0.1% Tween 20 at RT for 2 hr. The wells are then washed multiple times over 30 minutes. To each well is added 50 μL of polyclonal anti-phage antibody conjugated to horseradish peroxidase. The antibody is diluted 1:3000 in BSA-PBS-Tween 20; it was obtained from Pharmacia (Piscataway, NJ; catalog number 27-9402-01). After 30 minutes, the wells are washed again with BSA-PBS-Tween 20 for ~20 minutes. Finally, 100 μL of ABTS reagent (Pharmacia, with H_2O_2) are added to each well for the development of color. Plates are read with a plate reader (Molecular Devices, Menlo Park, CA) at 405 nm wavelength.

The nucleotide sequence of the relevant segments of the Src SH3 binding clones (or phage clones that bind to SH3 domains of other proteins) were sequenced using standard methods. Sanger, F., et al., in Proc. Natl. Acad. Sci. USA (1977) 74:5463-5467. The oligo primer 5'-AGCGTAACGATCTAAA-3' (SEQ ID NO:102) was used, which is 89 nucleotides downstream of the gene III cloning site of M13 m666. The nucleotide sequences were analyzed with the MacVector computer program (IBI, New Haven, CT, USA). From this nucleotide sequence information the primary sequence of each Src SH3 binding peptide was deduced. The corresponding synthetic peptides were then prepared by techniques well known in the art with or without flanking sequences. Indeed, these synthetic peptides have been shown to bind to SH3 domain targets, with those possessing the phage flanking amino acid residues exhibiting greater binding affinity.

6.7. *In Vitro* Peptide Binding Assays

Peptides were obtained from Research Genetics (Birmingham, AL), Chiron Mimotopes (Victoria, Australia), or synthesized by conventional techniques by Dr. J. Mark Carter of Cytogen Corporation (Princeton, NJ). Peptide purity was assessed by HPLC and/or mass spectrometry. Biotinylated

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peptides were synthesized with either a KSGSG (SEQ ID NO:103) or a GSGS (SEQ ID NO:104) peptide linker (a spacer) between the biotin and the N-terminus of the peptide. Binding experiments were performed as above, excepting the use of 10 μM peptide instead of phage. Bound biotinylated peptide was detected with streptavidin conjugated to alkaline phosphatase (Sigma Chemical Co., St. Louis, MO). After one hour incubation period at room temperature, the wells were washed, and a solution of 3 mM p-nitrophenyl-phosphate (US Biochemicals, Cleveland, OH) in 50 mM $NaCO_3$ (pH 9.8), and 50 mM $MgCl_2$ was added and color allowed to develop. Signals were read with an ELISA plate reader (Molecular Devices, Menlo Park, CA) at 405 nm wavelength. Binding experiments were performed in triplicate. The results are presented in Figures 7 and 8.

6.8. Peptide Competition of GST-SH3 Affinity Precipitations of Cell Lysates

Labeled proteins are prepared by incubating a culture of HeLa cells overnight with ≥100 μCi/mL ^{35}S-methionine. The cells are then washed and lysed with mild detergent. This mixture of radioactive proteins is incubated with Src-GST fusion protein that has been immobilized on glutathione-linked Sepharose beads (Pharmacia, Piscataway, NJ). After several hours of tumbling, the beads are pelleted gently by low-speed centrifugation, and the supernatant is discarded. The beads are then resuspended into a slurry in PBS-0.1% Tween 20, pelleted, and washed several additional times. Finally, a 2% SDS solution is added to the sample, which is then boiled at 100 °C for 3 minutes. Afterward, the sample is centrifuged, and the supernatant loaded on a 10% polyacrylamide SDS gel for electrophoresis. After the proteins have been resolved, the gel is fixed, dried down, and exposed to X-ray film for autoradiography or phosphor plates for scanning by a Molecular Dynamics PhosphorImager.

The ability of Src SH3 to bind certain ^{35}S-labeled proteins is examined for competability with exogenous

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peptides. Synthetic peptides corresponding to phage-displayed inserts and motifs are added at the time that the lysate is incubated with the Src-GST fusion protein immobilized on glutathione-linked sepharose beads. The SH3 binding peptides block binding of all or some of the labeled proteins while negative control peptides (unrelated peptide sequences) do not. The amount of competition is quantified and correlated with the amount of added SH3-domain binding peptides.

Alternatively, NIH 3T3 cells were grown in Dulbecco's Modified Eagle Medium (DME) + 10% fetal calf serum (FCS) + 80 μCi/mL Tran^{35}Slabel (ICN), washed with PBS, lysed in RIPA buffer, and pelleted. Supernatant from 1.5 x 10^6 cells was precleared with 100 μg glutathione-agarose-immobilized GST. The supernatant was then incubated with 10 μg glutathione-agarose-immobilized GST-SH3 fusion protein with or without added test peptide in a final volume of 250 μL. Pelleted beads were washed with 1 mL each of RIPA, RIPA + 1% deoxycholate + 0.1% SDS, and PBS, resuspended in 50 μL SDS-PAGE sample buffer, boiled, and subjected to SDS-PAGE (7.5%). Labeled proteins were detected by phosphorimaging (Molecular Dynamics). The results are presented in Figure 9.

6.9. Peptide Competition of GST-SH3 Affinity Precipitations of PI-3′ Kinase From Cell Lysates

It is possible to follow the precipitation of PI-3′ Kinase by Src from cell lysates in the presence or absence of SH3-binding peptides. HeLa cells are lysed with detergent and the protein mixtures are incubated for several hours with the Src-GST fusion protein immobilized on glutathione-linked Sepharose beads. After several hours of tumbling, the beads are pelleted gently by low-speed centrifugation and the supernatant is discarded. The beads are then resuspended into a slurry in PBS-0.1% Tween 20, pelleted, and washed several additional times. Finally, an SDS solution is added to the sample, which is then boiled at 100 °C for 3 minutes.

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Subsequently, the sample is centrifuged, and the supernatant is loaded on a 10% polyacrylamide SDS gel for electrophoresis. After the proteins have been resolved, the gel is blotted to nitrocellulose or nylon (i.e., western blot). The filter is then probed with a PI-3′ Kinase antibody (monoclonal and polyclonal antibodies are available from Upstate Biotechnology Incorporated, Lake Placid, NY) and an enzyme-linked secondary antibody. The amount of PI-3′ Kinase is then quantitated.

The ability of Src SH3 to bind PI-3′ Kinase is examined for competability with exogenous peptides. Synthetic peptides corresponding to phage-displayed inserts and motifs are added at the time that the lysate is incubated with the Src-GST fusion protein that has been immobilized on glutathione-linked sepharose beads. Ten-fold and one hundred-fold molar excess of peptides are used relative to SH3 proteins. The SH3 binding peptides block binding of the PI-3′ Kinase as detected on western blots while negative control peptides (unrelated peptide sequences) do not. The amount of competition is quantified and correlated with the amount of added SH3-domain binding peptides.

6.10. *In Vivo* Association Of SH3-Binding Peptides With SH3-Domains Of Proteins

To demonstrate association of the SH3-binding peptides with SH3-domains of proteins inside cells, the SH3-binding peptides are tagged and localized in cells. For example, Bar-Sagi et al., in Cell (1993) 74:83-91, have shown that SH3-binding proteins localize to the cytoskeleton when expressed in cells. Thus, the SH3 domain-binding peptides of the present invention can serve as cellular targetting signals (e.g., to the cytoskeleton). Accordingly, the peptides are tagged with biotin and, subsequently, injected into cells. Alternatively, one can transfect into cells a recombinant plasmid that expresses a fusion protein comprising of the SH3-binding peptide and the green fluorescent protein (GFP, Chalfie et al., in Science (1994)

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263:802-805). The location of the biotinylated peptide or the GFP fusion protein is then assayed with FITC-labeled streptavidin in paraformaldehyde-fixed cells or by direct fluorescence in living cells, respectively. Localization of the SH3-binding peptides to the cytoskeleton demonstrates that the SH3-binding peptides can bind SH3-domain proteins *in vivo*. In addition, focal adhesions, which are rich in Src, are also sites of potential subcellular localization of SH3-binding peptides.

Thus, NIH 3T3 fibroblasts were cultured *in vitro* on glass coverslips coated with fibronectin. After two days of growth at 37 °C, the cells were fixed for one hour at room temperature in the presence of 2% paraformaldehyde (pH 7.5). The coverslips were washed with PBS-0.1% Tween 20 several times to remove the fixative. Next, the coverslips were dipped into acetone (chilled at -20 °C) for approximately 20 seconds and allowed to air-dry. The coverslips were washed again with PBS-0.1% Tween 20, containing BSA (1 mg/mL), and incubated for 2 hours at room temperature with different biotinylated peptides in PBS-0.1% Tween 20. The coverslips were washed and then incubated with 1 mg/mL streptavidin-Cy3 (Jackson Immunoresearch Co., West Grove, PA) for 1 hour at room temperature. Finally, the coverslips were washed in PBS-0.1% Tween 20, mounted in a glycerol solution on a glass slide, and viewed with a Nikon Optiphot epifluorescence microscope and a 60x oil immersion lens.

The results are presented in Figure 11, in which panel A displays cells stained with the conjugate biotin-spacer-VLKRPLPIPPVTR (SEQ ID NO:64); panel B exhibits cells stained with the conjugate, biotin-spacer-GILAPPVPPRNTR (SEQ ID NO:63); panel C shows cells stained with the long consensus peptide, biotin-spacer-RSTPRPLPPLPTTR (SEQ ID NO:67); and panel D shows cells stained with the proline-rich vinculin peptide conjugate, biotin-spacer-LAPPKPPLPEGEV (SEQ ID NO:70). The "spacer" sequence is KSGSG (SEQ ID NO:103). As shown in Figure 11, the panels in which SH3 domain-binding peptides were used present a bright display of fluorescence

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activity that is in sharp contrast to the relatively "dark" features of panel D (non-SH3 domain binding vinculin segment). These results demonstrate further the ability of the SH3 domain-binding peptides of the present invention to localize to protein targets (e.g., Src and Src-related proteins) within cells and provide an image thereof.

6.11. *In Vivo* Modulation Of *Src* In Oocytes With SH3-Binding Peptides

When *Xenopus laevis* oocytes are injected with mRNA encoding deregulated Src, there are *dramatic* cytological and biochemical changes in the oocyte (Unger, T.F. and Steele, R.E., in Mol. Cell. Biol. (1992) 12:5485-5498). The applicants have obtained plasmids for generating wild type and deregulated Src mRNA, which are available from Dr. Robert Steele (University of California at Irvine). Synthetic SH3-binding peptides are injected into oocytes that have been previously injected with Src mRNA. The state of the cytoskeleton is inspected visually by observing the arrangement of cortical pigment granules under a dissecting microscope. The state of phosphorylation of several proteins is examined by western blotting with an anti-phosphotryosine monoclonal antibody (4G10; Upstate Biotechnology Incorporated), as described in Unger and Steele, above.

6.12. Progesterone-induced *X. laevis* Oocyte Maturation

Segments of adult ovary were removed surgically and incubated in 0.1% collagenase type D (Boehringer Mannheim, Indianapolis, IN) in Ca^{2+}-free OR2 (82.5 mM NaCl, 2.5 mM KCl, 1.0 mM $MgCl_2$, 1.0 mM Na_2HPO_4, 5.0 mM HEPES, and 3.8 mM NaOH, pH 7.6). Oocytes were then washed 3-5 times with OR2 containing 1.0 mM $CaCl_2$ and allowed to recover in OR2 overnight at 18 °C. Stage VI oocytes were injected with 40 nL of 100 mM peptide or water. After injection, the oocytes were placed in OR2 with 2 mg/mL progesterone (Sigma, St

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Louis, MO) and incubated at 20 °C. Oocytes were scored at hourly time points for germinal vesicle breakdown (GVBD).

Figure 10 presents the results of this experiment. As shown by the graph, oocytes injected with the SH3 domain-binding peptide VLKRPLPIPPVTR (SEQ ID NO:64) exhibit a faster rate of progesterone-induced germinal vesicle breakdown relative to oocytes that had been injected with water or with the proline-rich vinculin peptide, LAPPKPPLPEGEV (SEQ ID NO:70). These results parallel those of Unger and Steele, *supra*, wherein oocytes injected with deregulated or active Src RNA matured at a faster rate than oocytes injected with water or wild-type Src mRNA (See Figrure 3B of the Unger and Steele article).

The present results obtained with Src SH3 domain-binding peptides suggest that these peptides modulate the biochemical activity of "cellular" Src; in particular, it is proposed that at least some of the Src SH3 domain-binding peptides of the present invention upregulate the biochemical activity of "cellular" Src, which may be downregulated or inhibited in its normal state. Hence, the administration of the SH3 domain-binding peptides of the present invention can constitute a novel method of modulating the activity of Src or Src-related proteins. Specifically, certain of these peptides are able to activate Src-family proteins.

6.13. *In Vivo* Antagonism Of Src In Src Transformed Cells With SH3-Binding Peptides

The coding regions for SH3-binding peptides are cloned into vectors that direct their expression in animal cells. A bipartite gene is constructed, encoding a protein with *c-myc* epitope and SH3-binding peptide, which is transcribed from a strong constitutive promoter (e.g., SV40, CMV, HSV TK, calmodulin). The vector is introduced into either normal or Src-transformed cells via transfection (e.g., electroporation, calcium phosphate, liposomes, DEAE dextran). Transfected cells express the bipartite gene transiently in

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culture. To create stable transformed cell lines, the vector carries a selectable marker (e.g., neomycin resistance) or transfection is performed in the presence of excess plasmid carrying a selectable marker (e.g., neomycin resistance) and cells selected for the marker. Transfected cells are stained by immunofluorescence to detect expression of the bipartite protein. The hybridoma 9E10 secretes a monoclonal antibody that is highly specific for the *c-myc* epitope (EQKLISEEDLN [SEQ ID NO:105]; see, Evan, G.A. et al., in Mol. Cell. Biol. (1985) 5:3610-3616). This antibody is used in immunofluorescence experiments to demonstrate that the bipartite protein is expressed inside the cells, and in some cases, localized to subcellular structures enriched in SH3 domain bearing proteins.

There are several controls used in these experiments. First, cells are transfected with vectors that do not have the SH3-binding peptide coding region. Second, normal (non-transformed) cells are transfected. Third, cells transformed by oncogenes other than Src are used in the transfection experiments. Fourth, cells are stained with other monoclonal antibodies that do not recognize the *c-myc* epitope.

Transfected cells are examined for any changes in cell shape, behavior, and metabolism as a consequence of expressing the SH3 binding peptides. Cell shape is examined by phase contrast microscope at several times after transfection; in particular, the flatness of the cells, their adhesion to the substrate, and the degree of cell ruffling are monitored. Cell division rates, cell migration, and contact inhibition are also observed over time. Finally, the amount of phosphorylated tyrosine in transfected cells is quantitated by phosphoaminoacid analysis and with an anti-phosphotryosine monoclonal antibody (4G10; Upstate Biotechnology Incorporated) in western blotting experiments.

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6.14. Distinct Ligand Preferences of Various SH3 Domains

6.14.1. Preparation of PXXP (SEQ ID NO: 161) Biased Peptide Libraries

Using procedures similar to those described in Sections 6.1 and 6.4 and also described in Sparks, A. B., et al., in Methods in Enzymology, (1995) 255:498-509, oligonucleotide inserts were constructed according to the schematic provided in FIG. 12. The two synthetic oligonucleotides (5'-ctgtgcctcgagk(nnk)$_6$cca(nnk)$_2$cca(nnk)$_6$tctagacgtgtcagt-3' (SEQ ID NO:162) and 5'-actgacacgtctaga-3'(SEQ ID NO:163), where k=g+t and n=g+a+t+c) were annealed and filled in with Sequenase (Amersham, Arlington Heights, Il). The inserts were then digested with *Xho* I and *Xba* I and were ligated into gene III of the mBAX vector.

The mBAX vector was created by generating cloning sites in gene III of the M13mp18 vector (Messing, J., 1991, "Cloning in M13 phage or how to use biology at its best," *Gene* 100, 3-12) in the manner of Fowlkes et al., 1992, *Biotechniques* 13, 422-427. The mBAX vector displays a peptide sequence at the N-terminus of the mature pIII protein that encodes the epitope for the mouse monoclonal antibody 7E11 (see FIG. 13); it includes the stop codon TAG in the coding region, which is suppressed in *E. coli* carrying suppressor tRNA gene mutations known as *sup*E or *sup*F. There are no other stop codons in the mBAX genome. The mBAX vector also carries a segment of the alpha fragment of β-galactosidase; bacterial cells expressing the omega fragment of β-galactosidase that are infected by a bacteriophage that expresses the alpha fragment convert the clear XGal substrate into an insoluble blue precipitate. Thus, plaques of such bacteriophage on such cells appear blue.

Recombinant mBAX molecules can be distinguished easily from non-recombinant molecules due to the TAG codon in the *Xho*I - *Xba*I segment in gene III of mBAX. When recombinants are generated by replacing the *Xho* I - *Xba* I fragment with

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oligonucleotides encoding random peptides, the recombinants can be grown in bacteria with (*e.g.*, DH5αF') or without (*e.g.*, JS5) suppressor tRNA mutant genes. On the other hand, the non-recombinant mBAX molecules fail to produce plaques on bacterial lawns where the bacteria (*e.g.*, JS5) lack such suppressor genes. This is because in JS5, the TAG codon serves as a stop codon to yield a truncated pIII molecule during translation; since pIII is an essential protein component of viable M13 viral particles, no plaques will form.

The ligated DNA was electroporated into JS5 *E. coli* and recombinant phage were propagated on two hundred 100 mm 2xYT + 0.8% agar plates as described in Sambrook, J., Frisch, E., F., & Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Laboratory, Plainview, NY) (Sambrook et al.). To minimize the recovery of sibling clones during affinity purification of binding phage, six distinct library fractions were prepared by dividing the plates into six roughly equal groups. Each fraction was treated separately in all subsequent manipulations. Phage particles were harvested from each fraction by diffusion into 100 ml PBS (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.4 mM KH_2PO_4), concentrated by polyethylene glycol precipitation as in Sambrook et al. (1989, *supra*), and resuspended in 10 ml PBS + 10% glycerol. Each fraction contained approximately $5x10^7$ unique recombinants, for a total library complexity of approximately $3x10^8$. The resulting phage-displayed library contained peptides of the form X_6PXXPX_6 (SEQ ID NO:164), where X represents any amino acid.

6.14.2. Affinity purification of SH3-binding phage

Library screens were performed as described in Sparks, A. B., et al., in Methods in Enzymology, (1995) 255:498-509. Briefly, wells of an ELISA microtiter plate were coated with 10 μg GST-SH3 fusion protein in 100 mM $NaHCO_3$ (pH 8.5) for 3 hours and blocked with Superblock (Pierce, Rockford, IL) for

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1 hour. Approximately 5 x 10^{11} infectious particles from each library fraction were diluted in 200 μl PBS + 0.1% Tween 20 and incubated in a GST-SH3-coated well for 3 hours. The wells were washed five times with PBS + 0.1% Tween 20, and bound phage were eluted with 50 mM glycine-HCl (pH 2.2). Recovered phage were propagated in 10 ml 2xYT media and 100 μl of a saturated DH5αF′ *E. coli* culture and affinity purified twice more as above. Affinity purified phage were plated onto 2xYT + 0.8% agar plates to yield isolated plaques from which clonal phage stocks and DNA were produced. Phage binding was confirmed by incubating equal amounts of a clonal phage stock in wells coated with 1 μg GST-SH3 or GST. The wells were washed five times with PBS + 0.1% Tween 20, and bound phage were detected by anti-phage ELISA according to the manufacturer's instructions (Pharmacia, Piscataway, NJ). Clones with strong SH3-binding activity were selected for further analysis. The sequences of peptides displayed by these clones were determined by DNA sequencing of phage inserts.

6.14.3. Preparation of GST-SH3 fusion proteins

Constructs encoding GST fusions to the Grb2 N-terminal (Grb2 N, aa 1-58), Nck N-terminal (Nck N, aa 1-68), Nck middle (Nck M, aa 101-166), Nck C-terminal (Nck C, aa 191-257), p53bp2 (aa 454-530), or Src (aa 87-143) SH3 domains were generated by PCR cloning of the appropriate cDNAs into pGEX-2T (Pharmacia, Piscataway, NJ; a general reference for the pGEX vectors is Smith, D. B., & Johnson, K. S. (1988) *Gene* **67**, 31-40). The integrity of the constructs was confirmed by DNA sequencing. pGEX-derived constructs expressing GST fusions to the SH3 domains of Yes, Cortactin, Crk, Abl, and PLCγ were kindly provided by M. Sudol (Rockefeller University), J. T. Parsons (University of Virginia at Charlottsville), M. Matsuda (Tokyo, Japan), A. M. Pendergast (Duke University), and S. Earp (University of North Carolina at Chapel Hill), respectively. Alternatively,

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the GST-SH3 fusion proteins for Yes, Cortactin, Crk, Abl, and PLCγ could have been prepared as above for Grb2 N, Nck N, Nck M, Nck C, p53bp2, and Src, using published sequence information for these proteins. See, e.g., Suen et al., (1993) *Mol. Cell. Biol.* **13,** 5500-5512 (Grb2); Lehmann et al., (1990) *Nucleic Acids Res.* **18,** 1048 (Nck); Iwabuchi et al., (1994) *Proc. Natl. Acad. Sci. USA* **91,** 6098-6102 (p53bp2); Takeya et al., (1983) *Cell* **32,** 881-890 (Src); Sudol et al., (1988) *Nucleic Acids Res.* **16,** 9876 (Yes); Wu et al., (1991) *Mol. Cell. Biol.* **11,** 5113-5124 (Cortactin); Matsuda et al., (1992) *Mol. Cell. Biol.* **12,** 3482-3489 (Crk); Shtivelman et al., (1986) *Cell* **47,** 277-284 (Abl); Burgess et al., (1990) *Mol. Cell. Biol.* **10,** 4770-4777 (PLCγ). GST-SH3 fusion proteins were prepared as described in Smith, D. B., & Johnson, K. S. (1988) *Gene* **67,** 31-40. The integrity and purity of the fusion proteins were confirmed by SDS-PAGE. Protein concentrations were determined using a the BioRad protein assay (BioRad, Hercules, CA).

6.14.4. SH3 Domain Binding Peptides and Consensus Sequences

The use of second generation or biased peptide libraries, which fix all or part of the PXXP (SEQ ID NO:161) consensus motif for SH3 domain binding peptides and randomize flanking residues, has defined additional sequence residues exhibiting selective SH3 domain binding.

Tables 1-5, below, list some of the relevant amino acid sequences obtained when the biased peptide library described in Section 6.14.1 was screened with GST-SH3 fusion proteins. The underscored amino acid residues in Tables 1-5 indicate the fixed positions. Also, indicated for each set of new binders is a "consensus" sequence, which seeks to include the additional features gleaned from the new binding peptides. The symbol "ϕ" in the consensus sequences of Tables 1-5 represents a hydrophobic residue. The symbol x in the consensus sequences of Tables 1-5 represents any amino acid.

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For the Nck SH3 domain binding clones, a GST-SH3 fusion protein containing the middle SH3 domain of Nck was used.

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TABLE 1 CORTACTIN SH3-BINDING PEPTIDES

		SEQ. ID NO.
PXXP.CORT.M1/2/3.PP	SSLLGPPVPPKPQTLFSFSR	107
PXXP.CORT.M4.PP	SRLGEFSKPPIPQKPTWMSR	108
PXXP.CORT.N2.PP	SRTERPPLPQRPDWLSYSSR	109
PXXP.CORT.N3.PP.INC	SREPDWLCPNCPLLLRSDSR	110
PXXP.CORT.01/2/3.PP	SSSSHNSRPPLPEKPSWLSR	111
PXXP.CORT.04.PP	SRLTPQSKPPLPPKPSAVSR	112
CONSENSUS	KPPφPxKPxW R	113

BNSDOCID: <WO___9730074A1_I_>

TABLE 2 NCK SH3-BINDING PEPTIDES

		SEQ. ID NO.
PXXP.NCK.Q1/4.PP	SSLGVGWKPLPPMRTASLSR	114
PXXP.NCK.Q2/3.PP.INC	SSVGFADRPRPPLRVESLSR	115
PXXP.NCK.R1.PP.INC	SSAGILRPPEKPXRSFSLSR	116
PXXP.NCK.R2.PP	SSPYTGDVPIPPLRGASLSR	117
PXXP.NCK.R3.PP	SSLMGSWPPVPPLRSDSLSR	118
PXXP.NCK.R4.PP	SSIGEDTPPSPPTRRASLSR	119
PXXP.NCK.S1/4.PP	SRSLSEVSPKPPIRSVSLSR	120
PXXP.NCK.S2.PP.INC	SSVSEGYSPPLPPRSTSLSR	121
PXXP.NCK.S3.PP	SSSFTLAAPTPPTRSLSLSR	122
PXXP.NCK.T1.PP	SSPPYELPPRPPNRTVSLSR	123
PXXP.NCK.T2.PP	SRVVDGLAPPPPVRLSSLSR	124
PXXP.NCK.T3.PP.INC	SSLGYSGAPVPPHRxSSLSR	125
PXXP.NCK.T4.PP	SSISDYSRPPPPVRTLSLSR	126
CONSENSUS	ϕxxxxxPxPPϕRSxSL T	127

BNSDOCID: <WO___9730074A1_I_>

TABLE 3 ABL SH3 BINDING PEPTIDES

		SEQ. ID NO.
PXXP.ABL.G1/2.PP	SRGPRWSPPPVPLPTSLDSR	128
PXXP.ABL.G3/4.PP	SSPPDYAAPAIPSSLWVDSR	129
PXXP.ABL.H1/3/4.PP	SSPPHWAPPAPPAMSPPISR	130
PXXP.ABL.H2.PP.INC	SSDRCWECPPWPAGGQRGSR	131
PXXP.ABL.I1/2/3.PP	SSPPKFSPPPPPYWQLHASR	132
PXXP.ABL.I4.PP	SSPPSFAPPAAPPRHSFGSR	133
PXXP.ABL.J1.PP	SSAPKKPAPPVPMMAHVMSR	134
PXXP.ABL.J2.PP.INC	SSPTYPPPPPPDTAKGASR	135
PXXP.ABL.J3.PP.INC	SSPPXXXPPPIPNSPQVLSR	136
PXXP.ABL.J4.PP	SSPPTWTPPKPPGWGVVFSR	137
PXXP.ABL.L1.PP	SSAPTWSPPALPNVAKYKSR	138
PXXP.ABL.L2/3.PP	SSIKGPRFPVPPVPLNGVSR	139
PXXP.ABL.L4.PP	SSPPAWSPPHRPVAFGSTSR	140
CONSENSUS	PPxWxPPPφP	141

TABLE 4 PLCγ SH3-BINDING PEPTIDES

		SEQ. ID NO.
PXXP.PLCγ.P1.PP	SSMKVHNFPLPPLPSYETSR	142
PXXP.PLCγ.P2.PP	SRVPPLVAPRPPSTLNSLSR	143
PXXP.PLCγ.PE.PP.INC	SSLYWQHGPDPPVGAPQLSR	144
PXXP.PLCγ.P4.PP	SSHPLNSWPGGPFRHNLSSR	145

BNSDOCID: <WO___9730074A1_I_>

TABLE 5 SRC SH3-BINDING PEPTIDES

		SEQ. ID NO.
PXXP.SRC.A1.PP	SSRALRVRPLPPVPGTSLSR	146
PXXP.SRC.A2.PP	SSFRALPLPPTPDNPFAGSR	147
PXXP.SRC.A3.PP	SRDAPGSLPFRPLPPVPTSR	148
PXXP.SRC.A4.PP	SSISQRALPPLPLMSDPASR	149
PXXP.SRC.B1.PP	SSPAYRPLPRLPDLSVIYSR	150
PXXP.SRC.B2/3/PP	SSFINRRLPALPPDNSLLSR	151
PXXP.SRC.B4.PP	SRLTGRPLPALPPPFSDFSR	152
PXXP.SRC.C1.PP	SRMKDRVLPPIPTVESAVSR	153
PXXP.SRC.C2.PP.INC	SSLYSAIAPDPPPRNSSSSR	154
PXXP.SRC.C3.PP	SSLASRPLPLLPNSAPGQSR	155
PXXP.SRC.D1.PP	SSLTSRPLPDIPVRPSKSSR	156
PXXP.SRC.D2.PP.INC	SSLKWRALPPLPETDTPYSR	157
PXXP.SRC.D3.PP	SSNTNRLPPPTPDGLDVRSR	158
PXXP.SRC.D4.PP	SSLQSRPLPLPPQSSYPISR	159
CONSENSUS	RPLPPLP	9

In addition to the consensus sequence shown in Table 5, the amino acid sequences of the inserts from the Src SH3 domain-binding phage isolated from the PXXP (SEQ ID NO:161) biased peptide library described in Section 6.14.1 also give rise to the consensus sequence LXXRPLPXψP (SEQ ID NO:165), as shown in Table 6, below. In the consensus sequence LXXRPLPXψP (SEQ ID NO:165), ψ represents aliphatic amino acid residues (A, V, L, I, P); X represents any amino acid.

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TABLE 6

Src SH3 Binding Peptides

LASRPLPLLPNSAPGQ	(a portion of SEQ ID NO:155)
LTGRPLPALPPPFSDF	(a portion of SEQ ID NO:152)
PAYRPLPRLPDLSVIY	(a portion of SEQ ID NO:150)
RALRVRPLPPVPGTSL	(a portion of SEQ ID NO:146)
DAPGSLPFRPLPPVPT	(a portion of SEQ ID NO:148)
LKWRALPPLPETDTPY	(a portion of SEQ ID NO:157)
ISQRALPPLPLMSDPA	(a portion of SEQ ID NO:149)
LTSRPLPDIPVRPSKS	(a portion of SEQ ID NO:156)
NTNRPLPPTPDGLDVR	(a portion of SEQ ID NO:158)
MKDRVLPPIPTVESAV	(a portion of SEQ ID NO:153)
LQSRPLPLPPQSSYPI	(a portion of SEQ ID NO:159)
FINRRLPALPPDNSLL	(a portion of SEQ ID NO:151)
FRALPLPPTPDNPFAG	(a portion of SEQ ID NO:147)
LYSAIAPDPPPRNSSS♦	(a portion of SEQ ID NO:154)
LXXRPLPXψP = CONSENSUS	(SEQ. ID NO:165)

In Table 6, ψ represents aliphatic amino acid residues (A, V, L, I, P); X represents any amino acid; ♦ putative class II peptide (see Section 6.14.5). Invariant proline residues are underlined.

Another consensus sequence that can be derived from the amino acid sequences of the inserts from the Src SH3 domain-binding phage is:

$$LX_1X_2RPLPX_3\psi PX_4X_5 \text{ (SEQ ID NO:454)}$$

where ψ represents aliphatic amino acid residues (A, V, L, I, P) and X_1, X_2, X_3, X_4, and X_5 represent any amino acid; except that if

X_3 = P, ψ = L, X_4 = P, and X_5 = P, then:

where X_1 = F, then X_2 is not H or R; or

where X_1 = S, then X_2 is not R, H, A, N, T, G, V, M, or W; or

where X_1 = C, then X_2 is not S or G; or

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where X_1 = R, then X_2 is not T or F; or
where X_1 = A, then X_2 is not R, Q, N, S, or L; or
where X_1 = Q, then X_2 is not M; or
where X_1 = L, then X_2 is not R; or
where X_1 = I, then X_2 is not A; or
where X_1 = P, then X_2 is not P, W, or R; or
where X_1 = G, then X_2 is not S or R; or
where X_1 = T, then X_2 is not T.

In addition to the consensus sequence shown in Table 1, the amino acid sequences of the inserts from the cortactin SH3 domain-binding phage isolated from the PXXP (SEQ ID NO:161) biased peptide library described in Section 6.14.1 also give rise to the consensus sequence +PPψPXKPXWL (SEQ ID NO:166), as shown in Table 7, below.

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TABLE 7

Cortactin SH3 Binding P ptides

LTPQSKPPLPPKPSAV	(a portion of SEQ ID NO:112)
SSHNSRPPLPEKPSWL	(a portion of SEQ ID NO:111)
PVKPPLPAKPWWLPPL	(SEQ ID NO:167)
TERPPLPQRPDWLSYS	(a portion of SEQ ID NO:109)
LGEFSKPPIPQKPTWM	(a portion of SEQ ID NO:108)
YPQFRPPVPPKPSLMQ	(SEQ ID NO:168)
VTRPPLPPKPGHMADF	(SEQ ID NO:169)
VSLGLKPPVPPKPMQL	(SEQ ID NO:170)
LLGPPVPPKPQTLFSF	(a portion of SEQ ID NO:107)
YKPEVPARPIWLSEL	(SEQ ID NO:171)
GAGAARPLVPKKPLFL	(SEQ ID NO:172)
+PPψPXKPXWL = CONSENSUS	(SEQ ID NO:166)

In Table 7, + represents basic amino acid residues (R, K); ψ represents aliphatic amino acid residues (A, V, L, I, P); X represents any amino acid. Invariant proline residues are underlined.

In addition to the consensus sequence shown in Table 3, the amino acid sequences of the inserts from the Abl SH3 domain-binding phage isolated from the PXXP (SEQ ID NO:161) biased peptide library described in Section 6.14.1 also give rise to the consensus sequence PPXθXPPPψP (SEQ ID NO:173), as shown in Table 8, below.

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TABLE 8

Abl SH3 Binding P ptid s

PPWWAPPPIPNSPQVL	(SEQ ID NO:174)
PPKFSPPPPPYWQLHA	(a portion of SEQ ID NO:132)
PPHWAPPAPPAMSPPI	(a portion of SEQ ID NO:130)
PPTWTPPKPPGWGVVF	(a portion of SEQ ID NO:137)
PPSFAPPAAPPRHSFG	(a portion of SEQ ID NO:133)
PTYPPPPPPDTAKGA†	(a portion of SEQ ID NO:135)
GPRWSPPPVPLPTSLD	(a portion of SEQ ID NO:128)
APTWSPPALPNVAKYK	(a portion of SEQ ID NO:138)
PPDYAAPAIPSSLWVD	(a portion of SEQ ID NO:129)
IKGPRFPVPPVPLNGV	(a portion of SEQ ID NO:139)
PPAWSPPHRPVAFGST	(a portion of SEQ ID NO:140)
APKKPAPPVPMMAHVM	(a portion of SEQ ID NO:134)
PPXθXPPPψP = CONSENSUS	(SEQ ID NO:173)

In Table 8, θ represents aromatic amino acid residues; ψ represents aliphatic amino acid residues (A, V, L, I, P); X represents any amino acid. Invariant proline residues are underlined.

† This clone contained a three nucleotide deletion in the random peptide coding sequence.

The amino acid sequences of the inserts from the PLCγ SH3 domain-binding phage isolated from the PXXP (SEQ ID NO:161) biased peptide library described in Section 6.14.1 give rise to the consensus sequence PPVPPRPXXTL (SEQ ID NO:175), as shown in Table 9, below.

BNSDOCID: <WO___9730074A1_I_>

TABLE 9

PLCγ SH3 Binding Peptides

MPPPVPPRPPGTLQVA	(SEQ ID NO:176)
LSYSPPPVPPRPDSTL	(SEQ ID NO:177)
VLAPPVPPRPGNTFFT	(SEQ ID NO:178)
YRPPVAPRPPSSLSVD	(SEQ ID NO:179)
LQCPDCPRVPPRPIPI	(SEQ ID NO:180)
VPPLVAPRPPSTLNSL	(a portion of SEQ ID NO:143)
LTPPPFPKRPRWTLPE	(SEQ ID NO:181)
YWPHRPPLAPPQTTLG	(SEQ ID NO:182)
PPVPPRPXXTL = CONSENSUS	(SEQ ID NO:175)

In Table 9, the symbol X represents any amino acid. Invariant proline residues are underlined.

The PXXP (SEQ ID NO:161) biased peptide library described in Section 6.14.1 was also used to obtain phage clones that specifically bound the SH3 domain from the p53bp2 protein. The amino acid sequences of the peptides expressed by the p53bp2 SH3 domain-binding phage are shown in Table 10 below.

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TABLE 10

p53bp2 SH3 Binding P ptides

YDASSAPQRPPLPVRKSRP	(SEQ ID NO:183)
EYVNASPERPPIPGRKSRP	(SEQ ID NO:184)
WNGIAIPGRPEIPPRASRP	(SEQ ID NO:185)
SMIFIYPERPSPPPRFSRP	(SEQ ID NO:186)
GVEEWNPERPQIPLRLSRP	(SEQ ID NO:187)
WVVDSRPDIPLRRSLP	(SEQ ID NO:188)
VVPLGRPEIPLRKSLP	(SEQ ID NO:189)
GGTVGRPPIPERKSVD	(SEQ ID NO:190)
YSHAGRPEVPPRQSKP	(SEQ ID NO:191)
FSAAARPDIPSRASTP	(SEQ ID NO:192)
LYIPKRPEVPPRRHEA	(SEQ ID NO:193)
NNISARPPLPSRQNPP	(SEQ ID NO:194)
MAGTPRPAVPQRMNPP	(SEQ ID NO:195)
RPXψPψR+SXP = CONSENSUS	(SEQ ID NO:196)

In Table 10, + represents basic amino acid residues (R, K); ψ represents aliphatic amino acid residues (A, V, L, I, P); X represents any amino acid. Invariant proline or flanking residues are underlined.

The PXXP (SEQ ID NO:161) biased peptide library described in Section 6.14.1 was also used to obtain phage clones that specifically bound the SH3 domain from the N terminal portion of the Crk protein. The amino acid sequences of the peptides expressed by the Crk N terminal SH3 domain-binding phage are shown in Table 11 below.

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TABLE 11

Crk N SH3 Binding Peptides

GQPAGDPDPPPLPAKF	(SEQ ID NO:197)
FEQTGVPLLPPKSFKY	(SEQ ID NO:198)
IFGDPPPPIPMKGRSL	(SEQ ID NO:199)
SNQGSIPVLPIKRVQY	(SEQ ID NO:200)
NYVNALPPGPPLPAKN	(SEQ ID NO:201)
SSDPERPVLPPKLWSV	(SEQ ID NO:202)
HFGPSKPPLPIKTRIT	(SEQ ID NO:203)
DWKVPEPPVPKLPLKQ	(SEQ ID NO:204)
ATSEGLPILPSKVGSY	(SEQ ID NO:205)
NANVSAPRAPAFPVKT	(SEQ ID NO:206)
EMVLGPPVPPKRGTVV	(SEQ ID NO:207)
AGSRHPPTLPPKESGG	(SEQ ID NO:208)
SVAADPPRLPAKSRPQ	(SEQ ID NO:209)
ψPψLPψK = CONSENSUS	(SEQ ID NO:210)

In Table 11, ψ represents aliphatic amino acid residues (A, V, L, I, P). Invariant proline residues are underlined.

The present invention provides a purified peptide that binds to the SH3 domain of Crk, the purified peptide comprising the amino acid sequence ψPψLPψK (SEQ ID NO:210), where ψ represents aliphatic amino acid residues (A, V, L, I, P), with the proviso that the peptide does not comprise the amino acid sequence WNERQPAPALPPKPPKPT (SEQ ID NO:456).

The PXXP (SEQ ID NO:161) biased peptide library described in Section 6.14.1 was also used to obtain phage clones that specifically bound the SH3 domain from the Yes protein. The amino acid sequences of the peptides expressed by the Yes SH3 domain-binding phage are shown in Table 12 below.

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TABLE 12

Y s SH3 Binding Peptid s

ITMRPLPALPGHGQIH	(SEQ ID NO:211)
LPRRPLPDLPMAAGKG	(SEQ ID NO:212)
LGSRPLPPTPRQWPEV	(SEQ ID NO:213)
STIRPLPAIPRDTLLT	(SEQ ID NO:214)
RSGRPLPPIPEVGHNV	(SEQ ID NO:215)
IGSRPLPWTPDDLGSA	(SEQ ID NO:216)
LAQRELPGLPAGAGVS	(SEQ ID NO:217)
IPGRALPELPPQRALP	(SEQ ID NO:218)
FVGRELPPTPRTVIPW	(SEQ ID NO:219)
DPRSALPALPLTPLQT	(SEQ ID NO:220)
SPHDVLPALPDSHSKS	(SEQ ID NO:221)
ψXXRPLPXLP = CONSENSUS	(SEQ ID NO:222)

In Table 12, ψ represents aliphatic amino acid residues (A, V, L, I, P); X represents any amino acid. Invariant proline residues are underlined.

Another consensus sequence that can be derived from the amino acid sequences of the inserts from the Yes SH3 domain-binding phage is:

$$\psi X_1X_2RPLPX_3LPX_4X_5 \text{ (SEQ ID NO:455)}$$

where ψ represents aliphatic amino acid residues (A, V, L, I, P) and X_1, X_2, X_3, X_4, and X_5 represent any amino acid; except that if

X_3 = P, X_4 = P, and X_5 = P, then:

when ψ = L,

where X_1 = F, then X_2 is not H or R; or

where X_1 = S, then X_2 is not R, H, A, N, T, G, V, M, or W; or

where X_1 = C, then X_2 is not S or G; or

where X_1 = R, then X_2 is not T or F; or

where X_1 = A, then X_2 is not R, Q, N, S, or L; or

where X_1 = Q, then X_2 is not M; or

where X_1 = L, then X_2 is not R; or

BNSDOCID: <WO___9730074A1_I_>

where X_1 = I, then X_2 is not A; or
where X_1 = P, then X_2 is not P, W, or R; or
where X_1 = G, then X_2 is not S or R; or
where X_1 = T, then X_2 is not T; and
when ψ = P,
where X_1 = A, then X_2 is not R; or
where X_1 = S, then X_2 is not R or Y; or
where X_1 = M, then X_2 is not S; or
where X_1 = V, then X_2 is not G; or
where X_1 = R, then X_2 is not S; or
where X_1 = I, then X_2 is not R; and
when ψ = A,
where X_1 = A, then X_2 is not K; and
when ψ = V,
where X_1 = A, then X_2 is not C or Q; or
where X_1 = P, then X_2 is not P; and
when ψ = I,
where X_1 = G, then X_2 is not H; or
where X_1 = T, then X_2 is not S; or
where X_1 = R, then X_2 is not S.

The present invention also provides a purified peptide that binds to the SH3 domain of Yes, the purified peptide comprising the amino acid sequence ψX_1X_2RPLPX_3LPX_4X_5 (SEQ ID NO:455), where ψ represents aliphatic amino acid residues (A, V, L, I, P) and X_1, X_2, X_3, X_4, and X_5 represent any amino acid, with the proviso that the peptide does not comprise the amino acid sequence AGDRPLPPLPYNPKS (SEQ ID NO:457).

The PXXP (SEQ ID NO:161) biased peptide library described in Section 6.14.1 was also used to obtain phage clones that specifically bound the SH3 domain from the N terminal portion of the Grb2 protein. The amino acid sequences of the peptides expressed by the Grb2 N terminal SH3 domain-binding phage are shown in Table 13 below. These sequences can be arranged into three groups of sequences that have different, but related, consensus sequences. An overall

BNSDOCID: <WO 9730074A1 I_>

consensus sequence, +θDXPLPXLP (SEQ ID NO:223), can be derived for the three groups.

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TABLE 13

Grb2 N SH3 Binding Peptides

	KWDSLLPALPPAFTVE	(SEQ ID NO:224)
	RWDQVLPELPTSKGQI	(SEQ ID NO:225)
	RFDFPLPTHPNLQKAH	(SEQ ID NO:226)
	RLDSPLPALPPTVMQN	(SEQ ID NO:227)
	RWGAPLPPLPEYSWST	(SEQ ID NO:228)
	YWDMPLPRLPGEEPSL	(SEQ ID NO:229)
	RFDYNLPDVPLSLGTA	(SEQ ID NO:230)
	TKKPNAPLPPLPAYMG	(SEQ ID NO:231)
	KWDLDLPPEPMSLGNY	(SEQ ID NO:232)
	+θDXPLPXLP = CONSENSUS	(SEQ ID NO:223)
	YYQRPLPPLPLSHFES	(SEQ ID NO:234)
	YYRKPLPNLPRGQTDD	(SEQ ID NO:235)
	YFDKPLPESPGALMSL	(SEQ ID NO:236)
	YFSRALPGLPERQEAH	(SEQ ID NO:237)
	Yθ X+PLPXLP = CONSENSUS	(SEQ ID NO:238)
	SLWDPLPPIPQSKTSV	(SEQ ID NO:239)
	SYYDPLPKLPDPGDLG	(SEQ ID NO:240)
	KLYYPLPPVPFKDTKH	(SEQ ID NO:241)
	DPYDALPETPSMKASQ	(SEQ ID NO:242)
	θDPLPXLP = CONSENSUS	(SEQ ID NO:243)
	+θDXPLPXLP = OVERALL CONSENSUS	(SEQ ID NO:223)

In Table 13, + represents basic amino acid residues (R, K); θ represents aromatic amino acid residues; X represents any amino acid. Invariant proline residues are underlined.

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6.14.5. SH3 Ligand Binding Orientation

Peptide ligands bound to SH3 domains have been shown to assume a left-handed polyproline type II (PPII) helix conformation (Yu, H., Chen, J. K., Feng, S., Dalgarno, D. C., Brauer, A. W., & Schreiber, S. L. (1994) *Cell* **76**, 933-45). SH3 ligands are pseudo-symmetrical and may therefore bind in one of two opposite orientations (Feng, S., Chen, J. K., Yu, H., Simon, J. A., & Schreiber, S. L. (1994) *Science* **266**, 1241-7) (Feng et al.). Feng et al., *supra*, have demonstrated that peptides that bind in one or the other orientation share different consensus motifs. Specifically, ligands that bind in the Class I or Class II orientation conform to the consensus +pYPpYP (SEQ ID NO:244) or YPpYPp+ (SEQ ID NO:245) respectively, where uppercase positions represent conserved residues that contact the SH3 domain and confer specificity, and lowercase positions represent scaffolding residues that tend to be proline.

According to this model, we predict that the peptides selected by the Src, Yes, Abl, and Grb2 N SH3 domains bind in the Class I orientation, whereas the peptides selected by the Cortactin, p53bp2, PLCγ, and Crk N SH3 domains bind in the Class II orientation (see Table 14). Interestingly, most of the SH3 ligand consensus motifs identified in this work contain additional conserved residues flanking the SH3-binding core defined by Feng et al., *supra*. Furthermore, these conserved residues are situated N- and C-terminal of the SH3-binding core in Class I and Class II motifs, respectively, and are therefore predicted to interact with equivalent regions of their target SH3 domains (see Table 14).

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TABLE 14

Alignment of SH3 ligand consensus motifs

		SEQ ID NO:
Class I	**+pψPpψP**	244
Src	LXX**R**P**L**P**X**ψ**P**	165
Yes	ψXX**R**P**L**P**X**L**P**	222
Abl	P**P**X*θ*XP**P**P**P**ψ**P**	173
Grb2 N	+*θ*DX**P**L**P**X**L**P	223
	Y*θ*X**R**P**L**P**X**L**P**	246
	*θ*D**P**L**P**X**L**P	243
Class II	**ψPpψPp+**	245
Cortactin	+**P**P**P**ψ**P**X**K**P**X**W**L**	166
p53bp2	**R**P**X**ψ**P**ψ**R**+S**X**P	196
PLCγ	**XPPVPPRPXXTL**	247
Crk N	ψ**P**ψ**L**P**L**ψ**K**	210

In Table 14, each SH3 ligand consensus motif was assigned to class I or II based on its agreement with the class I or II consensus motif. Highly (>90%) conserved positions in each SH3 ligand consensus motif are listed in boldface and were interpreted as SH3 contact residues. + represents basic amino acid residues (K, R); ψ represents aliphatic amino acid residues (A, V, L, I, P); *θ* represents aromatic amino acid residues; X represents any amino acid; lower case p represents residues that tend to be proline.

The Src SH3 domain is capable of binding both Class I and Class II peptides Feng et al., *supra*. Although Class I peptides predominate in the population of Src SH3 ligands selected from the PXXP (SEQ ID NO:161) library, one clone conforms well to the Class II consensus (see Table 6). Previously, Sparks, A. B., Quilliam, L. A., Thorn, J. M., Der, C. J., & Kay, B. K. (1994) J. Biol. Chem. **269**, 23853-6 and Yu, H., Chen, J. K., Feng, S., Dalgarno, D. C., Brauer, A. W., & Schreiber, S. L. (1994) Cell **76**, 933-45 had isolated Class II Src SH3 ligands sharing the consensus PPψPPR (SEQ ID NO:248). Similarly, whereas the Grb2 N SH3 domain has been

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shown to bind peptides from SOS with the Class II consensus sequence PPψPPR (SEQ ID NO:248) (Rozakis-Adcock, M., Fernley, R., Wade, J., Pawson, T., & Bowtell, D. (1993) Nature **363,** 83-5), we have isolated Grb2 N SH3 ligands that conform to the Class I consensus (see Table 14). Thus, both the Src and the Grb2 N SH3 domains apparently have the capacity to bind both Class I and Class II peptide ligands.

6.14.6. SH3 Ligand Binding Characteristics

To explore further the capacity of SH3 domains to discriminate between different SH3 ligands, we investigated the binding of phage expressing various peptide ligands to a panel of SH3 domains. Equal titers of clonal phage stocks were incubated in microtiter wells coated with different GST-SH3 fusion proteins. The wells were washed several times, and bound phage were detected with an anti-phage antibody (see Fig. 14). Positive ELISA signals were equivalent to those obtained with previously characterized Src SH3-binding clones (Sparks, A. B., Quilliam, L. A., Thorn, J. M., Der, C. J., & Kay, B. K. (1994) J. Biol. Chem. **269,** 23853-6) and are indicative of SH3:peptide affinities in the 5 to 75 μM range (Yu, H., Chen, J. K., Feng, S., Dalgarno, D. C., Brauer, A. W., & Schreiber, S. L. (1994) Cell **76,** 933-945; Rickles, R. J., Botfield, M. C., Weng, Z., Taylor, J. A., Green, O. M., Brugge, J. S., & Zoller, M. J. (1994) EMBO J. **13,** 5598-604). Whereas the Src, Yes, Crk, and Grb2 N SH3 domains cross-reacted with a few phage clones selected with other SH3 domains, the Abl, Cortactin, p53bp2, and PLCγ SH3 domains displayed considerable specificity. Significantly, only 33 of 220 potential instances of cross-reactivity were observed, suggesting that SH3 selectivity is the rule rather than the exception.

Each instance of cross-reactivity may be explained by similarities between the sequences of the peptides and the ligand preferences of the cross-reactive SH3 domains. For example, Crk SH3 cross-reacted with three phage clones selected with other SH3 domains; each of these clones

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coincidentally expressed peptides conforming to the Crk SH3 preferred ligand consensus motif. Similarly, the cross-reactivity observed between the Src, Yes, and Grb2 SH3 domains and clones selected by other SH3 domains within this group may be a consequence of the fact that these SH3 domains prefer the same proline-rich core. Finally, the Src and Yes SH3 domains cross-reacted with the PLCγ SH3 ligand MPPPVPPRPPGTL (a portion of SEQ ID NO:176), which contains the Class II Src SH3-binding sequence PPVPPR (SEQ ID NO:249). Taken together, these data demonstrate the capacity of SH3 domains to discern subtle differences in the primary structure of potential ligands.

6.15. Use of Consensus Sequences to Determine the Amino Acid Sequences Responsible for Binding in Proteins that are Known to Bind SH3 Domains

There are many proteins that are known to bind SH3 domains but for which the specific sequences of those proteins that are responsible for binding to SH3 domains are not known. The consensus sequences shown above in Tables 1-13 can be used to search databases (*e.g.*, GenBank) containing the amino acid sequences of those proteins in order to determine which sequences are responsible for the binding of those proteins to SH3 domains. This was done for a number of known SH3 domain binding proteins and sequences resembling the consensus sequences of Tables 1-13 were identified. The results are shown in Table 15. For comparison, also shown in Table 15 are the amino acid sequences that had previously been demonstrated to be responsible for SH3 domain binding for a number of proteins.

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TABLE 15

			SEQ ID NO:	Reference
Src SH3 Class I		**LXXRPLPXψP**	165	
Hs AFAP-110	(62-73)	PPQMPLPEIPQQ	250	1
	(76-87)	PPDNGPPPLPTS	251	1
Hs CDC42 GAP	(250-261)	TAPKPMPPRPPL	252	2
Hs hnRNP K	(302-313)*	SRARNLPLPPPP	253	3
Mm p62	(328-339)	TVTRGVPPPPTV	254	3
Hs PI3K p85	(90-101)*	RPPRPLPVAPGS	255	9
Hs Shc p52	(296-307)	VRKQMLPPPPCP	256	3
Src SH3 Class II		**PPψPPR**	248	
Hs Dynamin	(810-820)	GGAPPVPSRPG	257	6
	(827-837)	GPPPQVPSRPN	258	6
	(838-848)	RAPPGVPSRSG	259	6
Hs hnRNP K	(308-318)*	PLPPPPPPRGG	260	3
Mm p62	(294-304)	APPPPPVPRGR	261	3
Hs Paxillin	(42-52)	AVPPPVPPPPS	262	10
Hs PI3K p85	(302-312)*	QPAPALPPKPP	263	9
Hs Shb	(50-60)	GGPPPGPGRRG	264	11
	(103-113)	TKSPPQPPRPD	265	11
Yes SH3		**ψXXRPLPXLP**	222	
Hs Yap65	(240-251)	PVKQPPPLAPQS	266	4
Abl SH3		**PPXθXPPPψP**	173	
Mm 3BP-1	(265-276)*	RAPTMPPPLPPV	267	12
Mm 3BP-2	(200-211)*	YPPAYPPPPVPV	268	12
Dm Ena	(350-361)	PGPGYGPPPVPP	269	5
PLCγ SH3		**PPVPPRPXXTL**	175	
Hs Dynamin	(812-823)	APPVPSRPGASP	270	6
	(829-840)	PPQVPSRPNRNR	271	6

BNSDOCID: <WO___9730074A1_I_>

			SEQ ID NO:	Reference
Hs c-Cbl	(493-504)	LPPVPPRLDLLP	272	7
Crk N SH3		**PψLPψK**	210	
Hs Abl	(524-533)	*QAPELPTKTR	273	13
	(568-577)	*VSPLLPRKER	274	13
	(758-767)	EKPALPRKRA	275	13
Hs C3G	(282-291)	*PPPALPPKKR	276	14
	(452-461)	*TPPALPEKKR	277	14
	(539-548)	*KPPPLPEKKN	278	14
	(607-616)	*PPPALPPKQR	279	14
Grb2 N SH3 Class I		**+θDXPLPXLP**	233	
		YθX+PLPXLP	238	
		θDPLPXLP	243	
Hs c-Cbl	(560-571)	PQRRPLPCTPGD	280	8
	(589-600)	WLPRPIPKVPVS	281	8
Grb2 N SH3 Class II		**PPPψPPR**	282	
Hs Abl	(523-533)*	LQAPELPTKTR	283	13
	(567-577)*	AVSPLLPRKER	284	13
	(609-619)*	KTAPTPPKRSS	285	13
Hs c-Cbl	(491-501)	ASLPPVPPRLD	286	8
Hs Dynamin	(810-820)	GGAPPVPSRPG	287	6
	(827-837)	GPPPQVPSRPN	288	6
	(838-848)	RAPPGVPSRSG	289	6
Hs SOS1	(1148-1158)*	PVPPPVPPRRR	290	15
	(1177-1187)	DSPPAIPPRQP	291	15
	(1209-1219)*	ESPPLLPPREP	292	15
	(1287-1297)*	IAGPPVPPRQS	293	15
Rn Synapsin I	(592-602)	NLPEPAPPRPS	294	16
	(670-680)	PPGPAGPIRQA	295	16

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In Table 15, + represents basic amino acid residues (R, K); ψ represents aliphatic amino acid residues (A, V, L, I, P); θ represents aromatic amino acid residues; X represents any amino acid. * represents amino acid sequences previously demonstrated to bind their respective SH3 domains. Residues within the sequences that agree with the most highly conserved residues of the consensus motifs are shown in bold. Each entry shows an abbreviation of the name of the SH3 domain binding protein and the species from which it was derived. The amino acid positions in the mature proteins of the sequences shown are indicated in parentheses. For more details, see the reference listed for each protein.

Reference 1 is Flynn, D. C., Leu, T. H., Reynolds, A. B., & Parsons, J. T. (1993) *Mol Cell Biol* **13,** 7892-7900.

Reference 2 is Barfod, E. T., Zheng, Y., Kuang, W, J., Hart, M. J., Evans, T., Cerione, R. A., & Ashkenazi, A. (1993) *J Biol Chem* **268,** 26059-62.

Reference 3 is Weng, Z., Thomas, S. M., Rickles, R. J., Taylor, J. A., Brauer, A. W., Seidel-Dugan, C., Michael, W. M., Dreyfuss, G., & Brugge, J. S. (1994) *Mol Cell Biol* **14,** 4509-21.

Reference 4 is Sudol, M. (1994) *Oncogene* **9,** 2145-52.

Reference 5 is Gertler, F. B., Comer, A. R., Juang, J. L., Ahern, S. M., Clark, M. J., Liebl, E. C., & Hoffmann, F. M. (1995) *Genes Dev* **9,** 521-33.

Reference 6 is Gout, I., Dhand, R., Hiles, I. D., Fry, M. J., Panayotou, G., Das, P., Truong, O., Totty, N. F., Hsuan, J., Booker, G, W. & et al. (1993) *Cell* **75,** 25-36.

Reference 7 is Rivero-Lezcano, O. M., Sameshima, J. H., Marcilla, A., & Robbins, K. C. (1994) *J Biol Chem* **269,** 17363-6.

Reference 8 is Odai, H., Sasaki, K., Iwamatsu, A., Hanazono, Y., Tanaka, T., Mitani, K., Yazaki, Y. & Hirai, H. (1995) *J Biol Chem* **270,** 10800-5.

Reference 9 is Kapeller, R., Prasad, K. V., Janssen, O., Hou, W., Schaffhausen, B. S., Rudd, C. E., & Cantley, L. C. (1994) *J Biol Chem* **269,** 1927-33.

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Reference 10 is Weng, Z., Taylor, J. A., Turner, C. E., Brugge, J. S., & Seidel-Dugan, C. (1993) *J Biol Chem* **268,** 14956-63.

Reference 11 is Karlsson, T., Songyang, Z., Landgren, E., Lavergne, C., Di-Fiore, P. P., Anafi, M., Pawson, T., Cantley, L. C., Claesson-Welsh, L., & Welsh, M. (1995) *Oncogene* **10,** 1475-83.

Reference 12 is Ren, R., Mayer, B. J., Cicchetti, P., & Baltimore, D. (1993) *Science* **259,** 1157-61.

Reference 13 is Ren, R., Ye, Z. S., & Baltimore, D. (1994) *Genes Dev* **8,** 783-95.

Reference 14 is Knudsen, B. S., Feller, S. M., & Hanafusa, H. (1994) *J Biol Chem* **269,** 32781-7.

Reference 15 is Rozakis-Adcock, M., Fernley, R., Wade, J., Pawson, T., & Bowtell, D. (1993) *Nature* **363,** 83-5.

Reference 16 is McPherson, P, S., Czernik, A, J., Chilcote, T, J., Onofri, F., Benfenati, F., Greengard, P., Schlessinger, J., & De-Camilli, P. (1994) *Proc Natl Acad Sci USA* **91,** 6486-90.

The sequences shown in Table 15 are useful in that they can be used as ligands in the assays for the identification of compounds that affect binding of SH3 domain-containing proteins and their ligands that is described above in Section 5.6.

6.16. Use of Consensus Sequences to Identify Amino Acid Sequences Resembling SH3 Domain-binding Sequences in Proteins that are Not Known to Bind SH3 Domains

The consensus sequences shown above in Tables 1-13 can be used to search databases (*e.g.*, GenBank) containing the amino acid sequences of proteins that are not known to bind to SH3 domains. In this way, a large number of proteins not previously suspected of containing amino acid sequences that bind SH3 domains have been shown to contain such sequences. The portions of the amino acid sequences of these proteins that resemble one or more of the consensus motifs of Tables 1-13 are shown below in Table 16. The SH3 domain-binding

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sequences of the proteins shown in Table 16 can be used as ligands in the assays for the identification of compounds that affect binding of SH3 domain-containing proteins and their ligands that are described above in Section 5.6.

BNSDOCID: <WO___9730074A1_I_>

TABLE 16

LOCUS	ACCESSION #'S			DESCRIPTION				SEQUENCE	SRC	SRC	ABL	COR	P53	PLC	GRB	CRK
ABL_DROME	P00522			TYROSINE-PROTEIN KINASE DASH/AB	DRO	132	146	LLQSRPLPHIPAGST (296)	1							
						1380	1395	QIQQKPAVPHKPPLND (297)				2				2
ABP1_YEAST	P15891			ACTIN BINDING PROTEIN	SAC	514	528	SSAAPPPPPRRATPE (298)		1				2		
ACES_HUMAN	P22303			ACETYLCHOLINESTERASE PRECURSOR	HOM	73	87	MGPRRFLPPEPKQPW (299)	2							
ACM4_HUMAN	P08173			MUSCARINIC ACETYLCHOLINE RECEPT	HOM	276	290	PPPALPPPPRPVADK (300)		3				2		
ACRO_HUMAN	P10323			ACROSIN PRECURSOR (EC 3.4.21.10	HOM	329	343	QPPPRPLPPRPPAAQ (301)	1	2				1		
AGIE_RAT	Q00900			DNA-BINDING PROTEIN AGIE-BP1 (A	RAT	642	656	PNLRRGLPQVPYFSL (302)	2							
ANDR_HUMAN	P10275			ANDROGEN RECEPTOR	HOM	368	385	ALAGPPPPPPPPHPHARI (303)								
AOFB_HUMAN	P27338			AMINE OXIDASE (FLAVIN-CONTAINING)	HOM	480	494	TFLERHLPSVPGLLR (304)	2							
AP2_HUMAN	P05549			TRANSCRIPTION FACTOR AP-2	HOM	52	68	DFQPPYFPPPPYQPIYPQ (305)			2					
ATF3_HUMAN	P18847			CYCLIC-AMP-DEPENDENT TRANSCRIPT	HOM	57	71	CFCHRPLPVPPGSLV (306)	1							
B1AR_HUMAN	P08588			BETA-1 ADRENERGIC RECEPTOR.	HOM	282	296	APAPPPGPPRPAAAA (307)		0				0		
B3AR_HUMAN	P13945			BETA-3 ADRENERGIC RECEPTOR.	HOM	361	375	CRCGRRLPPEPCAAA (308)	2							
BCL2_CHICK	Q00709			APOPTOSIS REGULATOR BCL-2	GAL	33	47	GEDRPPVPPAPAPAA (309)								
BNI1_YEAST	P41832			BNI1 PROTEIN (SYNTHETIC LETHAL	SAC	1242	1256	PPPPPH-VPAKLFGS (310)				4				0
CADM_MOUSE	P33146			MUSCLE-CADHERIN (M-CADHERIN)	MUS	645	659	PQPHPVLPTSPSDIA (311)	3							
CALR_PIG	P25117			CALCITONIN RECEPTOR PRECURSOR	SUS	14	28	IFLNRP.PVLPDSAD (312)	1							
CBL_HUMAN	P22681			PROTO-ONCOGENE C-CBL.	HOM	490	504	QASSLPPVPPRLDLLP (313)		1				1		
						536	555	PPTLRDLPPPPPPDRPYSVG (314)	7	2				3		
						559	573	RPQRRPLPCTPGDCP (315)	2							
CCB3_RABIT	Q02343			BRAIN CALCIUM CHANNEL BII-1 PR	ORY	19	33	SDQGRNLPGTPVPAS (316)	3							
						2100	2114	RHSRRQLPPVPPKPRPLL (317)	1			1		1		0
CCB4_RABIT	Q02344			BRAIN CALCIUM CHANNEL BII-2 PRO	ORY	19	33	SDQGRNLPGTPVPAS (318)	3							
CG2A_BOVIN	P30274			G2/MITOTIC-SPECIFIC CYCLIN A	BOS	56	70	NDEYVPVPPWKANNK (319)				5				
CIC1_RAT	P35524			CHLORIDE CHANNEL PROTEIN, SKELE	RAT	724	741	QTPTPPPPPPPPLPPQFP (320)								
CIK5_HUMAN	P22460			POTASSIUM CHANNEL PROTEIN KV1.5	HOM	60	74	DSGVRPLPPLPDPGV (321)	0							
						71	85	DPGVRPLPPLPEELP (322)	0							
CINC_RAT	P15389			SODIUM CHANNEL PROTEIN, CARDIAO	RAT	1723	1739	LNTGPPYCDPNLPNSNG (323)			3					
CPI2_RABIT	P00187			CYTOCHROME P450 IA2 (EC 1.14.14	ORY	238	252	FPILRYLPNRPLQRF (324)	3							
CP75_SOLME	P37120			CYTOCHROME P450 LXXVA (EC 1.14	SOL	30	44	SWRRRKLPPGPEGWP (325)	2							
CPC7_RAT	P05179			CYTOCHROME P450 IIC7 (EC 1.14	RAT	23	37	SSRRRKLPPGPTPLP (326)	2							
CPC8_HUMAN	P10631			CYTOCHROME P450 IIC8 (EC 1.14.	HOM	23	37	SCRRRKLPPGPTPLP (327)	2							
CPCK_MACFA	P33262			CYTOCHROME P450 IIC20 (EC 1.14	MAC	23	37	SSGRRKLPPGPTPLP (328)	2							

BNSDOCID: <WO___9730074A1_I_>

LOCUS	ACCESSION #'S			DESCRIPTION				SEQUENCE	SRC	SRC	ABL	COR	P53	PLC	GRB	CRK
CPCM_RAT	P19225			CYTOCHROME P450 IIC22 (EC 1.14	RAT	23	37	HHVRRKLPPGPTPLP (329)	2							
CPT7_MOUSE	P27786			CYTOCHROME P450 XVIIA1 (P450-C	MUS	25	39	AKFPRSLPFLPLVGS (330)	2							
CR2_MOUSE	P19070			COMPLEMENT RECEPTOR TYPE 2 PREC	MUS	22	38	NARKPYYSLPIVPGTVL (331)			3					
CTK1_YEAST	Q03957			CTD KINASE ALPHA SUBUNIT (EC 2	SAC	30	44	QSLARPPPPKRIRTD (332)		3			1	3		
CXA3_BOVIN	P41987			GAP JUNCTION ALPHA-3 PROTEIN	BOS	287	301	ASPARALPGPPHPRR (333)	2	3				3		
CYA3_RAT	P21932			ADENYLATE CYCLASE, OLFACTIVE TY	RAT	829	843	TDSRLPLVPSKYSMT (334)				4				1
CYGR_HUMAN	Q02846			RETINAL GUANYLYL CYCLASE PRECUR	HOM	15	31	GLCGPAWWAPSLPRLPR (335)			3					
CYLI_HUMAN	P35663			CYLICIN (FRAGMENT).	HOM	571	587	LCWCKMPPPPPKPRYAP (336)				2		3		2
CYRG_MOUSE	P34902			CYTOKINE RECEPTOR COMMON GAMMA	MUS	283	298	WLERMPPIPPPKNLED (337)				5				2
DCD_HUMAN	P20711			AROMATIC-L-AMINO-ACID DECARBOXY	HOM	31	47	PDVEPGYLRPLIPAAAP (338)			3					
DMD_HUMAN	P11532			DYSTROPHIN	HOM	700	714	QEELPPPPPQKKRQI (339)	1							
DPOD_BOVIN	P28339			DNA POLYMERASE DELTA CATALYTIC	BOS	104	118	VAPARPLPGAPPPSQ (340)	1							
DRA_HUMAN	P40879			DRA PROTEIN (DOWN-REGULATED IN	HOM	319	335	GDMNPGFQPPITPDVET (341)			3					
DYI5_DROME	P13496			150 KD DYNEIN-ASSOCIATED POLYPE	DRO	1250	1264	ARSARRLPSWPPTLD (342)	3							
DYN1_HUMAN	Q05193			DYNAMIN-1	HOM	809	823	LGCAPPVPSRPGASP (343)		1				1		
E75C_DROME	P13055			ECDYSONE-INDUCIBLE PROTEIN E75-	DRO	398	413	VMRPPPPPPPPKVKHA (344)	3			3				1
						587	601	MRHGPGLPSTPCHTS (345)	3							
EGR2_HUMAN	P11161			EARLY GROWTH RESPONSE PROTEIN	HOM	113	127	HLPGPPPPPPPYSGS (346)								
ELK1_MOUSE	P41969			PROTEIN ELK-1 (FRAGMENT)	MUS	164	178	PQPQPPIPPRPASVL (347)		1				1		
ENL_HUMAN	Q03111			ENL PROTEIN.	HOM	272	286	PPPPPPPPPRASSKR (348)		1				2		
						452	467	LPSREPPPPQKPPPN (349)				2				
EPI5_HUMAN	P42566			EPIDERMAL GROWTH FACTOR RECEPTOR	HOM	763	778	KSEDEPPALPPKIGTP (350)				3				0
ERB3_HUMAN	P21860			ERBB-3 RECEPTOR PROTEIN-TYROSIN	HOM	1204	1218	RRHSPPHPPRPSSLE (351)	4	2				1		
EZRI_HUMAN	P15311	P23714		EZRIN (P81) (CYTOVILLIN) (VILLI	HOM	465	479	VMTAPPPPPPPVYEP (352)								
FAK_HUMAN	Q05397			FOCAL ADHESION KINASE (EC 2.7.1	HOM	183	197	KEGERALPSIPKLAN (353)	2							
FASL_MOUSE	P41047			FAS ANTIGEN LIGAND	MUS	41	55	DQRRPPPPPPPVSPL (354)	3							
FGR_FSVGR	P00544			TYROSINE-PROTEINKINASE TRANSFO	FEL	9	23	VCRPRPLPPLPPTAM (355)	0							
FOR4_MOUSE	Q05859			FORMIN 4 (LIMB DEFORMITY PROTEIN	MUS	655	669	PPLIPPPPPLPPGLG (356)								
						681	700	CPVSPPPPPPPPPPTPVPPS (357)								
						699	718	PSDGPPPPPPPPPPLPNVLA (358)								
						721	740	NSGGPPPPPPPPPPPPGLAP (359)								
FOSB_MOUSE	P13346			FOSB PROTEIN	MUS	253	269	GWLLPPPPPPLPFQSS (360)								
FOSB_CHICK	P11939			P55-C-FOS PROTO-ONCOGENE PROTEIN	GAL	239	254	LMTEAPPAVPPKEPSG (361)				3				0
FSH_DROME	P13709	P13710		FEMALE STERILE HOMEOTIC PROTEIN	DRO	4	20	SEPPPRYEPPVEPVNGI (362)			2					
G33_RATE	P05432			GENE 33 POLYPEPTIDE	RAT	146	160	DRSSRPLPPLPISED (363)	0							

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LOCUS	ACCESSION #'S			DESCRIPTION				SEQUENCE	SRC	SRC	ABL	COR	P53	PLC	GRB	CRK
						281	295	IPPRVPIPPRPAKPD (364)		3		3		1		3
GLI3_HUMAN	P10071			GLI3 PROTEIN	HOM	789	804	MFPRLNPILPPKAPAV (365)	4			3				1
						986	1000	AAPPRLLPPLPTCYG (366)	1							
GTPA_BOVIN	P09851			GTPASE-ACTIVATIGN PROTEIN (GAP)	BOS	127	141	GGGFPPLPPPPPQLP (367)								
HME1_MOUSE	P09065			HOMEOBOX PROTEIN ENGRAILED-1 (M	MUS	72	91	LPHPPPPPPPPPPPPQHLA (368)								
HMOC_DROME	P22810			HOMEOTIC PROTEIN ORTHODENTICLE	DRO	453	467	SAPQRPMPPNRPSPP (369)		4			1	2		
HS27_HUMAN	P04792			HEAT SHOCK 27 KD PROTEIN (HSP 2	HOM	48	64	GSSWPGYVRPLPPAAIE (370)			4					
HXA4_CHICK	P17277			HOMEOBOX PROTEIN HOX-A4 (CHOX-1	GAL	42	59	HPHAPPPPPPPPPPHLHA (371)								
						127	141	GASPPPPPPAKGHPG (372)				3				5
HXAA_HUMAN	P31260			HOMEOBOX PROTEIN HOX-A10 (HOX-1	HOM	723	737	PQQQPPPPPQPPQPA (373)								
HXB2_HUMAN	P14652	P17485	P10913	HOMEOBOX PROTEIN HOX-B2 (HOX-2H	HOM	75	91	GPALPPPPPPPLPAAPP (374)								
HXB3_HUMAN	P14651	P17484		HOMEOBOX PROTEIN HOX-B3 (HOX-2G	HOM	280	296	HSMTPSYESPSPPAFGK (375)			4					
HXB4_HUMAN	P17483			HOMEOBOX PROTEIN HOX-B4 (HOX-2	HOM	69	91	RDPGPPPPPPPPPPPPPPGLSP (376)								
HXC4_HUMAN	P09017			HOMEOBOX PROTEIN HOX-C4 (HOX-3	HOM	50	64	QELYPPPPPRPSYPE (377)						1		
IBP1_BOVIN	P24591			INSULIN-LIKE GROWTH FACTOR BIND	BOX	83	97	GLSCRALPGEPRPLH (378)	3							
IDE_HUMAN	P14735			INSULIN-DEGRADING ENZYME (EC 3	HOM	995	1009	TEFKRGLPI FPLVKP (379)	3							
IEF5_HUMAN	P31948			TRANSFORMATION-SENSITIVE PROTEI	HOM	195	211	EIATPPPPPPPKKETKP (380)				3				2
IHBB_RAT	P17491			INHIBIN BETA B CHAIN PRECURSOR	RAT	35	49	SPAAPHPPPPGAPG (381)								
IRS1_HUMAN	P35568			INSULIN RECEPTOR SUBSTRATE-1 (I	HOM	1197	1211	PEPQPPPPPPHQPL (382)								
ISP3_SCHPO	P40899			SEXUAL DIFFERENTIATION PROCESS	SCH	39	55	QHQQPTYWYPPPPRHH (383)		2	3			2		
JUND_CHICK	P27921			TRANSCRIPTION FACTOR JUN-D	GAL	203	218	PRLPPPPPPPLKDEPQ (384)	4			4				2
KICH_HUMAN	P35790			CHOLINE KINASE (EC 2.7.1.32)	HOM	53	67	ALALPPPPPLPLPLP (385)								
KU5_YEAST	P40494			PROBABLE SERINE/THREONINE-PROTE	SAC	744	759	KDKSRPPRPPPKPLHL (386)				2				
KIR1_HUMAN	Q04771			SERINE/THREONINE-PROTEIN KINASE	HOM	450	464	VDQQRPNIPNRWFSD (387)		3			1			
KIR4_HUMAN	P36897			SERINE/THREONINE-PROTEIN KINASE	HOM	447	461	EQKLRPNIPNRWQSC (388)					1			
KRAF_CAEEL	Q07292			RAF HOMOLOG SERINE/THREONINE-P	CAE	458	473	LDAQRPRPPQKPHHED (389)				2				
MAPA_RAT	P34926			MICROTUBULE-ASSOCIATED PROTEIN	RAT	1812	1826	VPKDRPLPPAPLSPA (390)	0							
						2421	2437	GELSPSFLNPPLPPSTD (391)			2					
MAPB_MOUSE	P14873			MICROTUBULE-ASSOCIATED PROTEIN	MUS	520	535	DLTGQVPTPPVKQVKL (392)				5				
MIS_HUMAN	P03971			MUELLERIAN INHIBITING FACTOR	HOM	266	280	LDTVPFPPPRPSAEL (393)						2		
						387	401	AAELRSLPGLPPATA (394)	2							
MPK1_XENLA	Q05116			DUAL SPECIFICITY MITOGEN-ACTIVA	XEN	286	300	ELAPRPRPPGRPISS (395)		3			0	3		
MPK2_HUMAN	P36507			DUAL SPECIFICITY MITOGEN-ACTIVA	HOM	293	307	SISPRPRPPGRPVSG (396)		3			0	3		
MYBB_CHICK	Q03237			MYB-RELATED PROTEIN B	GAL	512	526	YGPIRPLPQTPHLEE (397)	2							
MYSA_CAEEL	P12844			MYOSINE HEAVY CHAIN A (MHC A)	CAE	561	577	LGKHPNFQKPKPPKGKQ (398)			4					

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LOCUS	ACCESSION #'S			DESCRIPTION				SEQUENCE	SRC	SRC	ABL	COR	P53	PLC	GRB	CRK
MYSB_CAEEL	P02566			MYOSINE HEAVY CHAIN B (MHC B)	CAE	559	575	LGKHPNFEKPKPPKGKQ (399)			4					
MYSC_CAEEL	P12844			MYOSINE HEAVY CHAIN C (MHC C)	CAE	562	578	LGKHPNFEKPKPPKGKQ (400)			4					
MYSD_CAEEL	P02567			MYOSINE HEAVY CHAIN D (MHC D)	CAE	556	572	LGKHPNFEKPKPPKGKQ (401)			4					
NCF1_HUMAN	P14598			NEUTROPHIL CYTOSOL FACTOR 1 (N	HOM	359	373	SKPQPAVPPRPSADL (402)		2				1		
NEU_RAT	P06494			NEU ONCOGENE PRECURSOR (EC 2.7.	RAT	560	574	VSDKRCLPCHPECQP (403)	3							
NG3_DROME	P40140			NEW-GLUE PROTEIN 3 PRECURSOR (	DRO	33	47	LRLPPPLPPRPRQPL (404)		0				0		
NME4_MOUSE	Q03391			GLUTAMATE (NMDA) RECEPTOR SUBU	MUS	901	915	PPAKPPPPPQPLPSP (405)								
OIF_HUMAN	P50774			OSTEOINDUCTIVE FACTOR PRECURSOR	HOM	177	192	NQLLKLPVLPPKLTLF (406)				3				
PI1B_HUMAN	P42338			PHOSPHATIDYLINOSITOL 3-KINASE (	HOM	309	323	SNLPLPLPPKKTRH (407)				4				
P2B1_HUMAN	P16298			SERINE/THREONINE PROTEIN PHOSPH	HOM	7	25	ARAAPPPPPPPPPPPGADR (408)	3							
P53_CHICK	P10360			CELLULAR TUMOR ANTIGEN P53.	GAL	45	62	EPSDPPPPPPPPLPLAA (409)								
P85A_HUMAN	P27986			PHOSPHATIDYLINOSITOL 3-KINASE	HOM	89	103	PRPPRPLPVAPGSSK (410)	1							
P85B_BOVIN	P23726			PHOSPHATIDYLINOSITOL 3-KINASE	BOS	90	105	PRGPRPLPPARPRDGP (411)	2	3			0	3		
						290	305	EQEVAPPALPPKPPKT (412)				2				0
PFTA_RAT	Q04631			PROTEIN FARNESYLTRANSFERASE AL	RAT	18	34	QPEQPPPPPPPPPAQGP (413)								
PRGR_HUMAN	P06401			PROGESTERONE RECEPTOR (PR) (FOR	HOM	419	433	LGPPPPLPPKATPSR (414)		0				1		
PRO_DROME	P29617			PROTEIN PROSPERO.	DRO	1076	1090	YHPQPPPPPPPMMPV (415)								
PRPB_HUMAN	P02814			PROLINE-RICH PEPTIDE P-B.	HOM	17	33	QPEGPGPEVPPPPPPPYG (416)			2					
PTN1_HUMAN	P18031			PROTEIN-TYROSINE PHOSPHATASE 1	HOM	302	316	PPERPPPPRPPKRI (417)		3		3		1		2
PTN3_HUMAN	P26045			PROTEIN-TYROSINE PHOSPHATASE P	HOM	850	874	CLTERNLPYPLDIV (418)	3							
PTN4_HUMAN	P29074			PROTEIN-TYROSINE PHOSPHATASE ME	HOM	457	472	PGDGKPPALPPKQSKK (419)				3				
PTP1_DROME	P35992			PROTEIN-TYROSINE PHOSPHATASE 10	DRO	1430	1446	PETWPDFGVPNPPQTLV (420)			4					
PTPK_MOUSE	P35822			PROTEIN-TYROSINE PHOSPHATASE KA	MUS	60	76	SAQEPSIYLPPEMPQGSY (421)			2					
RADI_HUMAN	P35241			RADIXIN.	HOM	466	481	VMSAPPPPPPPPVIPP (422)								
RB_HUMAN	P06400			RETINOBLASTOMA-ASSOCIATED PROTE	HOM	19	33	EPPAPPPPPPPEEDP (423)								
ROG_HUMAN	P38159			HETEROGENEOUS NUCLEAR RIBONUCLE	HOM	97	106	GRRGPPPPPPSRGPP (424)	4	1				2		
ROK_HUMAN	Q07244			HETEROGENEOUS NUCLEAR RIBONUCLE	HOM	267	281	GRGGRPMPPSRRDYD (425)		3			1			
					HOM	301	321	GSRARNLPLPPPPPPRGGDL (426)	3	1				1		
ROL_HUMAN	P14866			HETEROGENEOUS NUCLEAR RIBONUCLE	HOM	326	346	SRYGPQYGHPPPPPPPPEYGP (427)			3					
RRG1_HUMAN	P13631			RETINOIC ACID RECEPTOR GAMMA-1	HOM	76	90	SSPSPPPPPRVYKPC (428)		2				2		
RRG2_HUMAN	P22932			RETINOIC ACID RECEPTOR GAMMA-2	HOM	65	79	SSPSPPPPPRVYKPC (429)		2				2		
RRXB_HUMAN	P28702			RETINOIC ACID RECEPTOR RXR-BETA	HOM	95	109	GSGAPPPPPMPPPPL (430)								
RRXC_HUMAN	P28703			RETINOIC ACID RECEPTOR RXR-BETA		115	129	GSGAPPPPPMPPPPL (431)								
RYNR_HUMAN	P21817			RYANODINE RECEPTOR, SKELETAL MU	HOM	4516	4531	PKKQAPPSPPPKKEEA (432)				4				
SHC_HUMAN	P29353			SHC TRANSFORMING PROTEINS 46.8	HOM	297	311	RKQMPPPPPCPGREL (433)								

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LOCUS	ACCESSION #'S			DESCRIPTION				SEQUENCE	SRC	SRC	ABL	COR	P53	PLC	GRB	CRK
SLPI_DROME	P32030			FORK HEAD DOMAIN TRANSCRIPTION	DRO	242	258	GAPAPSYGYPAVPFAAA (434)			3					
SOS_DROME	P36675			SON OF SEVENLESS PROTEIN	DRO	1339	1353	RAVPPPLPPRRKERT (435)		0				1		
						1377	1391	ELSPPPIPPRLNHST (436)		0				1		
ST20_YEAST	Q03497			SERINE/THREONINE-PROTEIN KINASE	SAC	533	547	EQPLPPIPPTKSKTS (437)								
SUF_DROME	P25991			SUPPRESSOR OF FORKED PROTEIN.	DRO	229	243	KGLNRNLPAVPPTLT (438)	2							
SXLF_DROME	P19339			SEX-LETHAL PROTEIN, FEMALE-SPEC	DRO	308	322	PANVPPPPPQPPAHM (439)								
TACT_HUMAN	P40200			T-CELL SURFACE PROTEIN TACTILE	HOM	538	553	PPPFKPPPPPIKYTCI (440)			1	4				
TGFB_HUMAN	P22064			TRANSFORMING GROWTH FACTOR BETA	HOM	440	454	KSTHPPPLPAKEEPV (441)				3				
TIE2_MOUSE	Q02858			TYROSINE-PROTEIN KINASE RECEPTOR	MUS	725	739	SHELRTLPHSPASAD (442)	3							
TJ6_MOUSE	P15920			IMMUNE SUPPRESSOR FACTOR J6B7.	MUS	81	96	EGEASPPAPPLKHVLE (443)								
TLL_DROME	P18102			TAILLESS PROTEIN	DRO	214	228	ALATRALPPTPPLMA (444)	2							
TOP1_HUMAN	P11387			DNA TOPOISOMERASE I (EC 5.99.1.	HOM	221	237	EHKGPVFAPPYEPLPEN (445)			3					
TOPA_HUMAN	P11388			DNA TOPOISOMERASE II, ALPHA ISO	HOM	833	849	QRVEPEWYIPIIPMVLI (446)			3					
TOPB_HUMAN	Q02880			DNA TOPOISOMERASE II, BETA ISOZ	HOM	855	871	QRVEPEWYIPIIPMVLI (447)			3					
TRA1_HUMAN	P34708			SEX-DETERMINING TRANSFORMER PRO	CAE	1069	1090	PEDDPIYALPPPPPPPAPPRRR (448)			3					
TRT1_HUMAN	P13805			TROPONIN T, SLOW SKELETAL MUSCLE	HOM	42	57	SRPVVPPLIPPKIPEG (449)				3				
XA1_XENLA	P23507			XA-1 PROTEIN PRECURSOR.	XEN	23	39	GEDSPVFRPPSPPMGPS (450)			2					
						121	136	FRTGRPLLPIKPEHGR (451)				3				
ZO1_HUMAN	Q07157			TIGHT JUNCTION PROTEIN ZO-1.	HOM	1410	1424	IQATPPPPPLPSQYA (452)								
ZYX_CHICK	Q04584			ZYXIN.	GAL	120	134	AFPSPPPPPPPMFDE (453)								

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In Table 16, locus and accession number refer to the entries' names and accession numbers in GenBank or the Swiss-Prot database. The two numbers immediately to the left of the displayed sequences refer to the amino acid positions of the displayed sequences in the mature proteins. The leftmost of these two numbers refers to the starting amino acid number of the displayed sequence in the mature protein. The numbers in parentheses immediately to the right of the displayed sequences refer to the sequences' SEQ ID NOs:. The eight columns to the extreme right of Table 16 show the discrepancies between the displayed sequences and the consensus motifs of Tables 6-15. The leftmost Src column refers to Class I motifs; the rightmost Src column refers to Class II motifs.

It should be apparent to one of ordinary skill that many other embodiments of the present invention can be contemplated beyond the preferred embodiments described above but which other embodiments nevertheless fall within the scope and spirit of the present invention. Hence, the present invention should not be construed to be limited to the preferred embodiments described herein, which serve only to illustrate the present invention, but only by the claims that follow.

Also, numerous references are cited throughout the specification. The complete disclosures of these references are incorporated by reference herein.

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WHAT IS CLAIMED IS:

1. A purified peptide that binds to the SH3 domain of Cortactin, said peptide comprising the amino acid sequence ZPPϕPxKPxW (SEQ ID NO:113), where Z represents K or R; ϕ represents a hydrophobic amino acid; and x represents any amino acid.

2. A purified peptide that binds to the middle SH3 domain of Nck, said peptide comprising the amino acid sequence ϕxxxxxPxPPϕRZxSL (SEQ ID NO:127), where Z represents S or T; ϕ represents a hydrophobic amino acid; and x represents any amino acid.

3. A purified peptide that binds to the SH3 domain of Abl, said peptide comprising the amino acid sequence PPxWxPPPϕP (SEQ ID NO:141), where ϕ represents a hydrophobic amino acid; and x represents any amino acid.

4. A purified peptide that binds to the SH3 domain of Src, said peptide comprising the amino acid sequence LXXRPLPXψP (SEQ ID NO:165), where ψ represents an aliphatic amino acid; and X represents any amino acid.

5. A purified peptide that binds to the SH3 domain of Cortactin, said peptide comprising the amino acid sequence +PPψPXKPXWL (SEQ ID NO:166), where + represents a basic amino acid; ψ represents an aliphatic amino acid; and X represents any amino acid.

6. A purified peptide that binds to the SH3 domain of Abl, said peptide comprising the amino acid sequence PPXθXPPPψP (SEQ ID NO:173), where θ represents an aromatic amino acid; ψ represents an aliphatic amino acid; and X represents any amino acid.

7. A purified peptide that binds to the SH3 domain of PLCγ, said peptide comprising the amino acid sequence PPVPPRPXXTL (SEQ ID NO:175), where X represents any amino acid.

8. A purified peptide that binds to the SH3 domain of p53bp2, said peptide comprising the amino acid sequence RPXψPψR+SXP (SEQ ID NO:196), where + represents a basic amino

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acid; ψ represents an aliphatic amino acid; and X represents any amino acid.

9. A purified peptide that binds to the N terminal SH3 domain of Crk, said peptide comprising the amino acid sequence ψPψLPψK (SEQ ID NO:210), where ψ represents an aliphatic amino acid; and X represents any amino acid.

10. A purified peptide that binds to the SH3 domain of Yes, said peptide comprising the amino acid sequence ψXXRPLPXLP (SEQ ID NO:222), where ψ represents an aliphatic amino acid; and X represents any amino acid.

11. A purified peptide that binds to the N terminal SH3 domain of Grb2, said peptide comprising an amino acid sequence selected from the group consisting of: +θDXPLPXLP (SEQ ID NO:223), YθX+PLPXLP (SEQ ID NO:238), and θDPLPXLP (SEQ ID NO:243), where θ represent an aromatic amino acid; + represents a basic amino acid; ψ represents an aliphatic amino acid; and X represents any amino acid.

12. A purified peptide that binds to the SH3 domain of Cortactin, said peptide comprising an amino acid sequence selected from the group consisting of:
LTPQSKPPLPPKPSAV (a portion of SEQ ID NO:112);
SSHNSRPPLPEKPSWL (a portion of SEQ ID NO:111);
PVKPPLPAKPWWLPPL (SEQ ID NO:167);
TERPPLPQRPDWLSYS (a portion of SEQ ID NO:109);
LGEFSKPPIPQKPTWM (a portion of SEQ ID NO:108);
YPQFRPPVPPKPSLMQ (SEQ ID NO:168);
VTRPPLPPKPGHMADF (SEQ ID NO:169);
VSLGLKPPVPPKPMQL (SEQ ID NO:170);
LLGPPVPPKPQTLFSF (a portion of SEQ ID NO:107);
YKPEVPARPIWLSEL (SEQ ID NO:171);
GAGAARPLVPKKPLFL (SEQ ID NO:172); and
SREPDWLCPNCPLLLRSDSR (SEQ ID NO:110).

13. A purified peptide that binds to the middle SH3 domain of Nck, said peptide comprising an amino acid sequence selected from the group consisting of:
SSLGVGWKPLPPMRTASLSR (SEQ ID NO:114);
SSVGFADRPRPPLRVESLSR (SEQ ID NO:115);

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SSAGILRPPEKPXRSFSLSR (SEQ ID NO:116);
SSPYTGDVPIPPLRGASLSR (SEQ ID NO:117);
SSLMGSWPPVPPLRSDSLSR (SEQ ID NO:118);
SSIGEDTPPSPPTRRASLSR (SEQ ID NO:119);
SRSLSEVSPKPPIRSVSLSR (SEQ ID NO:120);
SSVSEGYSPPLPPRSTSLSR (SEQ ID NO:121);
SSSFTLAAPTPPTRSLSLSR (SEQ ID NO:122);
SSPPYELPPRPPNRTVSLSR (SEQ ID NO:123);
SRVVDGLAPPPPVRLSSLSR (SEQ ID NO:124);
SSLGYSGAPVPPHRxSSLSR (SEQ ID NO:125); and
SSISDYSRPPPPVRTLSLSR (SEQ ID NO:126).

14. A purified peptide that binds to the SH3 domain of Abl, said peptide comprising an amino acid sequence selected from the group consisting of:
PPWWAPPPIPNSPQVL (SEQ ID NO:174);
PPKFSPPPPPYWQLHA (a portion of SEQ ID NO:132);
PPHWAPPAPPAMSPPI (a portion of SEQ ID NO:130);
PPTWTPPKPPGWGVVF (a portion of SEQ ID NO:137);
PPSFAPPAAPPRHSFG (a portion of SEQ ID NO:133);
PTYPPPPPPDTAKGA (a portion of SEQ ID NO:135);
GPRWSPPPVPLPTSLD (a portion of SEQ ID NO:128);
APTWSPPALPNVAKYK (a portion of SEQ ID NO:138);
PPDYAAPAIPSSLWVD (a portion of SEQ ID NO:129);
IKGPRFPVPPVPLNGV (a portion of SEQ ID NO:139);
PPAWSPPHRPVAFGST (a portion of SEQ ID NO:140);
APKKPAPPVPMMAHVM (a portion of SEQ ID NO:134);
SSDRCWECPPWPAGGQRGSR (SEQ ID NO:131); and
SSPPXXXPPPIPNSPQVLSR (SEQ ID NO:136).

15. A purified peptide that binds to the SH3 domain of PLCγ, said peptide comprising an amino acid sequence selected from the group consisting of:
MPPPVPPRPPGTLQVA (SEQ ID NO:176);
LSYSPPPVPPRPDSTL (SEQ ID NO:177);
VLAPPVPPRPGNTFFT (SEQ ID NO:178);
YRPPVAPRPPSSLSVD (SEQ ID NO:179);
LQCPDCPRVPPRPIPI (SEQ ID NO:180);
VPPLVAPRPPSTLNSL (a portion of SEQ ID NO:143);

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LTPPPFPKRPRWTLPE (SEQ ID NO:181);
YWPHRPPLAPPQTTLG (SEQ ID NO:182);
SSMKVHNFPLPPLPSYETSR (SEQ ID NO:142);
SSLYWQHGPDPPVGAPQLSR (SEQ ID NO:144); and
SSHPLNSWPGGPFRHNLSSR (SEQ ID NO:145).

16. A purified peptide that binds to the SH3 domain of Src, said peptide comprising an amino acid sequence selected from the group consisting of:
LASRPLPLLPNSAPGQ (a portion of SEQ ID NO:155);
LTGRPLPALPPPFSDF (a portion of SEQ ID NO:152);
PAYRPLPRLPDLSVIY (a portion of SEQ ID NO:150);
RALRVRPLPPVPGTSL (a portion of SEQ ID NO:146);
DAPGSLPFRPLPPVPT (a portion of SEQ ID NO:148);
LKWRALPPLPETDTPY (a portion of SEQ ID NO:157);
ISQRALPPLPLMSDPA (a portion of SEQ ID NO:149);
LTSRPLPDIPVRPSKS (a portion of SEQ ID NO:156);
NTNRPLPPTPDGILDVR (a portion of SEQ ID NO:158);
MKDRVLPPIPTVESAV (a portion of SEQ ID NO:153);
LQSRPLPLPPQSSYPI (a portion of SEQ ID NO:159);
FINRRLPALPPDNSLL (a portion of SEQ ID NO:151);
FRALPLPPTPDNPFAG (a portion of SEQ ID NO:147); and
LYSAIAPDPPPRNSSS (a portion of SEQ ID NO:154).

17. A purified peptide that binds to the SH3 domain of p53bp2, said peptide comprising an amino acid sequence selected from the group consisting of:
YDASSAPQRPPLPVRKSRP SEQ ID NO:183);
EYVNASPERPPIPGRKSRP (SEQ ID NO:184);
WNGIAIPGRPEIPPRASRP SEQ ID NO:185);
SMIFIYPERPSPPPRFSRP (SEQ ID NO:186);
GVEEWNPERPQIPLRLSRP (SEQ ID NO:187);
WVVDSRPDIPLRRSLP (SEQ ID NO:188);
VVPLGRPEIPLRKSLP (SEQ ID NO:189);
GGTVGRPPIPERKSVD (SEQ ID NO:190);
YSHAGRPEVPPRQSKP (SEQ ID NO:191);
FSAAARPDIPSRASTP (SEQ ID NO:192);
LYIPKRPEVPPRRHEA (SEQ ID NO:193);
NNISARPPLPSRQNPP (SEQ ID NO:194); and

BNSDOCID: <WO__9730074A1_L>

MAGTPRPAVPQRMNPP (SEQ ID NO:195).

18. A purified peptide that binds to the N terminal SH3 domain of Crk, said peptide comprising an amino acid sequence selected from the group consisting of:

GQPAGDPDPPPLPAKF (SEQ ID NO:197);
FEQTGVPLLPPKSFKY (SEQ ID NO:198);
IFGDPPPPIPMKGRSL (SEQ ID NO:199);
SNQGSIPVLPIKRVQY (SEQ ID NO:200);
NYVNALPPGPPLPAKN (SEQ ID NO:201);
SSDPERPVLPPKLWSV (SEQ ID NO:202);
HFGPSKPPLPIKTRIT (SEQ ID NO:203);
DWKVPEPPVPKLPLKQ (SEQ ID NO:204);
ATSEGLPILPSKVGSY (SEQ ID NO:205);
NANVSAPRAPAFPVKT (SEQ ID NO:206);
EMVLGPPVPPKRGTVV (SEQ ID NO:207);
AGSRHPPTLPPKESGG (SEQ ID NO:208); and
SVAADPPRLPAKSRPQ (SEQ ID NO:209).

19. A purified peptide that binds to the SH3 domain of Yes, said peptide comprising an amino acid sequence selected from the group consisting of:

ITMRPLPALPGHGQIH (SEQ ID NO:211);
LPRRPLPDLPMAAGKG (SEQ ID NO:212);
LGSRPLPPTPRQWPEV (SEQ ID NO:213);
STIRPLPAIPRDTLLT (SEQ ID NO:214);
RSGRPLPPIPEVGHNV (SEQ ID NO:215);
IGSRPLPWTPDDLGSA (SEQ ID NO:216);
LAQRELPGLPAGAGVS (SEQ ID NO:217);
IPGRALPELPPQRALP (SEQ ID NO:218);
FVGRELPPTPRTVIPW (SEQ ID NO:219);
DPRSALPALPLTPLQT (SEQ ID NO:220); and
SPHDVLPALPDSHSKS (SEQ ID NO:221).

20. A purified peptide that binds to the N terminal SH3 domain of Grb2, said peptide comprising an amino acid sequence selected from the group consisting of:

KWDSLLPALPPAFTVE (SEQ ID NO:224);
RWDQVLPELPTSKGQI (SEQ ID NO:225);
RFDFPLPTHPNLQKAH (SEQ ID NO:226);

BNSDOCID: <WO___9730074A1_I_>

RLDSPLPALPPTVMQN (SEQ ID NO:227);
RWGAPLPPLPEYSWST (SEQ ID NO:228);
YWDMPLPRLPGEEPSL (SEQ ID NO:229);
RFDYNLPDVPLSLGTA (SEQ ID NO:230);
TKKPNAPLPPLPAYMG (SEQ ID NO:231);
KWDLDLPPEPMSLGNY (SEQ ID NO:232);
YYQRPLPPLPLSHFES (SEQ ID NO:234);
YYRKPLPNLPRGQTDD (SEQ ID NO:235);
YFDKPLPESPGALMSL (SEQ ID NO:236);
YFSRALPGLPERQEAH (SEQ ID NO:237);
SLWDPLPPIPQSKTSV (SEQ ID NO:239);
SYYDPLPKLPDPGDLG (SEQ ID NO:240);
KLYYPLPPVPFKDTKH (SEQ ID NO:241); and
DPYDALPETPSMKASQ (SEQ ID NO:242).

21. A purified peptide having an amino acid sequence selected from the group consisting of: SEQ ID NOs: 250-252, 254, 256-259, 261, 262, 264-266, 269-272, 275, 280, 281, 286-289, 291, 294, and 295.

22. A purified peptide having an amino acid sequence selected from the group consisting of: SEQ ID NOs: 296-453.

23. A method of identifying an inhibitor of the binding between a first molecule comprising an SH3 domain and a second molecule that binds to the SH3 domain comprising incubating one or more compounds from which it is desired to select such an inhibitor with the first molecule and the second molecule under conditions conducive to binding and detecting the one or more compounds that inhibit binding of the first molecule to the second molecule.

24. The method of claim 23 where the second molecule is obtained by:

(i) screening a peptide library with the SH3 domain to obtain peptides that bind the SH3 domain;

(ii) determining a consensus sequence for the peptides obtained in step (i);

(iii) producing a peptide comprising the consensus sequence;

BNSDOCID: <WO___9730074A1_I_>

wherein the second molecule comprises the peptide comprising the consensus sequence.

25. The method of claim 23 where the second molecule is obtained by:

(i) screening a peptide library with the SH3 domain to obtain peptides that bind the SH3 domain;

(ii) determining a consensus sequence for the peptides obtained in step (i);

(iii) searching a database to identify amino acid sequences that resemble the consensus sequence of step (ii);

(iv) producing a peptide comprising an amino acid sequence identified in step (iii);

wherein the second molecule comprises the peptide comprising an amino acid sequence identified in step (iii).

26. The method of claim 23 where the second molecule is a peptide that binds to the SH3 domain of Cortactin, said peptide comprising the amino acid sequence ZPPϕPxKPxW (SEQ ID NO:113), where Z represents K or R; ϕ represents a hydrophobic amino acid; and x represents any amino acid.

27. The method of claim 23 where the second molecule is a peptide that binds to the middle SH3 domain of Nck, said peptide comprising the amino acid sequence ϕxxxxxPxPPϕRZxSL (SEQ ID NO:127), where Z represents S or T; ϕ represents a hydrophobic amino acid; and x represents any amino acid.

28. The method of claim 23 where the second molecule is a peptide that binds to the SH3 domain of Abl, said peptide comprising the amino acid sequence PPxWxPPPϕP (SEQ ID NO:141), where ϕ represents a hydrophobic amino acid; and x represents any amino acid.

29. The method of claim 23 where the second molecule is a peptide that binds to the SH3 domain of Src, said peptide comprising the amino acid sequence LXXRPLPXψP (SEQ ID NO:165), where ψ represents an aliphatic amino acid; and X represents any amino acid.

30. The method of claim 23 where the second molecule is a peptide that binds to the SH3 domain of Cortactin, said peptide comprising the amino acid sequence +PPψPXKPXWL (SEQ

RNSDOCID: <WO 9730074A1_I >

ID NO:166), where + represents a basic amino acid; ψ represents an aliphatic amino acid; and X represents any amino acid.

31. The method of claim 23 where the second molecule is a peptide that binds to the SH3 domain of Abl, said peptide comprising the amino acid sequence PPXθXPPPψP (SEQ ID NO:173), where θ represents an aromatic amino acid; ψ represents an aliphatic amino acid; and X represents any amino acid.

32. The method of claim 23 where the second molecule is a peptide that binds to the SH3 domain of PLCγ, said peptide comprising the amino acid sequence PPVPPRPXXTL (SEQ ID NO:175), where X represents any amino acid.

33. The method of claim 23 where the second molecule is a peptide that binds to the SH3 domain of p53bp2, said peptide comprising the amino acid sequence RPXψPψR+SXP (SEQ ID NO:196), where + represents a basic amino acid; ψ represents an aliphatic amino acid; and X represents any amino acid.

34. The method of claim 23 where the second molecule is a peptide that binds to the N terminal SH3 domain of Crk, said peptide comprising the amino acid sequence ψPψLPψK (SEQ ID NO:210), where ψ represents an aliphatic amino acid; and X represents any amino acid.

35. The method of claim 23 where the second molecule is a peptide that binds to the SH3 domain of Yes, said peptide comprising the amino acid sequence ψXXRPLPXLP (SEQ ID NO:222), where ψ represents an aliphatic amino acid; and X represents any amino acid.

36. The method of claim 23 where the second molecule is a peptide that binds to the N terminal SH3 domain of Grb2, said peptide comprising an amino acid sequence selected from the group consisting of: +θDXPLPXLP (SEQ ID NO:223), YθX+PLPXLP (SEQ ID NO:238), and θDPLPXLP (SEQ ID NO:243), where θ represent an aromatic amino acid; + represents a basic amino acid; ψ represents an aliphatic amino acid; and X represents any amino acid.

BNSDOCID: <WO___9730074A1_I_>

37. The method of claim 23 where the second molecule is a peptide that binds to the SH3 domain of Cortactin, said peptide comprising an amino acid sequence selected from the group consisting of:

LTPQSKPPLPPKPSAV (a portion of SEQ ID NO:112);
SSHNSRPPLPEKPSWL (a portion of SEQ ID NO:111);
PVKPPLPAKPWWLPPL (SEQ ID NO:167);
TERPPLPQRPDWLSYS (a portion of SEQ ID NO:109);
LGEFSKPPIPQKPTWM (a portion of SEQ ID NO:108);
YPQFRPPVPPKPSLMQ (SEQ ID NO:168);
VTRPPLPPKPGHMADF (SEQ ID NO:169);
VSLGLKPPVPPKPMQL (SEQ ID NO:170);
LLGPPVPPKPQTLFSF (a portion of SEQ ID NO:107);
YKPEVPARPIWLSEL (SEQ ID NO:171);
GAGAARPLVPKKPLFL (SEQ ID NO:172); and
SREPDWLCPNCPLLLRSDSR (SEQ ID NO:110).

38. The method of claim 23 where the second molecule is a peptide that binds to the middle SH3 domain of Nck, said peptide comprising an amino acid sequence selected from the group consisting of:

SSLGVGWKPLPPMRTASLSR (SEQ ID NO:114);
SSVGFADRPRPPLRVESLSR (SEQ ID NO:115);
SSAGILRPPEKPXRSFSLSR (SEQ ID NO:116);
SSPYTGDVPIPPLRGASLSR (SEQ ID NO:117);
SSLMGSWPPVPPLRSDSLSR (SEQ ID NO:118);
SSIGEDTPPSPPTRRASLSR (SEQ ID NO:119);
SRSLSEVSPKPPIRSVSLSR (SEQ ID NO:120);
SSVSEGYSPPLPPRSTSLSR (SEQ ID NO:121);
SSSFTLAAPTPPTRSLSLSR (SEQ ID NO:122);
SSPPYELPPRPPNRTVSLSR (SEQ ID NO:123);
SRVVDGLAPPPPVRLSSLSR (SEQ ID NO:124);
SSLGYSGAPVPPHRxSSLSR (SEQ ID NO:125); and
SSISDYSRPPPPVRTLSLSR (SEQ ID NO:126).

39. The method of claim 23 where the second molecule is a peptide that binds to the SH3 domain of Abl, said peptide comprising an amino acid sequence selected from the group consisting of:

BNSDOCID: <WO__9730074A1_I_>

PPWWAPPPIPNSPQVL (SEQ ID NO:174);
PPKFSPPPPPYWQLHA (a portion of SEQ ID NO:132);
PPHWAPPAPPAMSPPI (a portion of SEQ ID NO:130);
PPTWTPPKPPGWGVVF (a portion of SEQ ID NO:137);
PPSFAPPAAPPRHSFG (a portion of SEQ ID NO:133);
PTYPPPPPPDTAKGA (a portion of SEQ ID NO:135);
GPRWSPPPVPLPTSLD (a portion of SEQ ID NO:128);
APTWSPPALPNVAKYK (a portion of SEQ ID NO:138);
PPDYAAPAIPSSLWVD (a portion of SEQ ID NO:129);
IKGPRFPVPPVPLNGV (a portion of SEQ ID NO:139);
PPAWSPPHRPVAFGST (a portion of SEQ ID NO:140);
APKKPAPPVPMMAHVM (a portion of SEQ ID NO:134);
SSDRCWECPPWPAGGQRGSR (SEQ ID NO:131); and
SSPPXXXPPPIPNSPQVLSR (SEQ ID NO:136).

40. The method of claim 23 where the second molecule is a peptide that binds to the SH3 domain of PLCγ, said peptide comprising an amino acid sequence selected from the group consisting of:
MPPPVPPRPPGTLQVA (SEQ ID NO:176);
LSYSPPPVPPRPDSTL (SEQ ID NO:177);
VLAPPVPPRPGNTFFT (SEQ ID NO:178);
YRPPVAPRPPSSLSVD (SEQ ID NO:179);
LQCPDCPRVPPRPIPI (SEQ ID NO:180);
VPPLVAPRPPSTLNSL (a portion of SEQ ID NO:143);
LTPPPFPKRPRWTLPE (SEQ ID NO:181);
YWPHRPPLAPPQTTLG (SEQ ID NO:182);
SSMKVHNFPLPPLPSYETSR (SEQ ID NO:142);
SSLYWQHGPDPPVGAPQLSR (SEQ ID NO:144); and
SSHPLNSWPGGPFRHNLSSR (SEQ ID NO:145).

41. The method of claim 23 where the second molecule is a peptide that binds to the SH3 domain of Src, said peptide comprising an amino acid sequence selected from the group consisting of:
LASRPLPLLPNSAPGQ (a portion of SEQ ID NO:155);
LTGRPLPALPPPFSDF (a portion of SEQ ID NO:152);
PAYRPLPRLPDLSVIY (a portion of SEQ ID NO:150);
RALRVRPLPPVPGTSL (a portion of SEQ ID NO:146);

BNSDOCID: <WO___9730074A1_I_>

DAPGSLPFRPLPPVPT (a portion of SEQ ID NO:148);
LKWRALPPLPETDTPY (a portion of SEQ ID NO:157);
ISQRALPPLPLMSDPA (a portion of SEQ ID NO:149);
LTSRPLPDIPVRPSKS (a portion of SEQ ID NO:156);
NTNRPLPPTPDGLDVR (a portion of SEQ ID NO:158);
MKDRVLPPIPTVESAV (a portion of SEQ ID NO:153);
LQSRPLPLPPQSSYPI (a portion of SEQ ID NO:159);
FINRRLPALPPDNSLL (a portion of SEQ ID NO:151);
FRALPLPPTPDNPFAG (a portion of SEQ ID NO:147); and
LYSAIAPDPPPRNSSS (a portion of SEQ ID NO:154).

42. The method of claim 23 where the second molecule is a peptide that binds to the SH3 domain of p53bp2, said peptide comprising an amino acid sequence selected from the group consisting of:

YDASSAPQRPPLPVRKSRP SEQ ID NO:183);
EYVNASPERPPIPGRKSRP (SEQ ID NO:184);
WNGIAIPGRPEIPPRASRP SEQ ID NO:185);
SMIFIYPERPSPPPRFSRP (SEQ ID NO:186);
GVEEWNPERPQIPLRLSRP (SEQ ID NO:187);
WVVDSRPDIPLRRSLP (SEQ ID NO:188);
VVPLGRPEIPLRKSLP (SEQ ID NO:189);
GGTVGRPPIPERKSVD (SEQ ID NO:190);
YSHAGRPEVPPRQSKP (SEQ ID NO:191);
FSAAARPDIPSRASTP (SEQ ID NO:192);
LYIPKRPEVPPRRHEA (SEQ ID NO:193);
NNISARPPLPSRQNPP (SEQ ID NO:194); and
MAGTPRPAVPQRMNPP (SEQ ID NO:195).

43. The method of claim 23 where the second molecule is a peptide that binds to the N terminal SH3 domain of Crk, said peptide comprising an amino acid sequence selected from the group consisting of:

GQPAGDPDPPPLPAKF (SEQ ID NO:197);
FEQTGVPLLPPKSFKY (SEQ ID NO:198);
IFGDPPPPIPMKGRSL (SEQ ID NO:199);
SNQGSIPVLPIKRVQY (SEQ ID NO:200);
NYVNALPPGPPLPAKN (SEQ ID NO:201);
SSDPERPVLPPKLWSV (SEQ ID NO:202);

BNSDOCID: <WO__9730074A1_I_>

HFGPSKPPLPIKTRIT (SEQ ID NO:203);
DWKVPEPPVPKLPLKQ (SEQ ID NO:204);
ATSEGLPILPSKVGSY (SEQ ID NO:205);
NANVSAPRAPAFPVKT (SEQ ID NO:206);
EMVLGPPVPPKRGTVV (SEQ ID NO:207);
AGSRHPPTLPPKESGG (SEQ ID NO:208); and
SVAADPPRLPAKSRPQ (SEQ ID NO:209).

44. The method of claim 23 where the second molecule is a peptide that binds to the SH3 domain of Yes, said peptide comprising an amino acid sequence selected from the group consisting of:
ITMRPLPALPGHGQIH (SEQ ID NO:211);
LPRRPLPDLPMAAGKG (SEQ ID NO:212);
LGSRPLPPTPRQWPEV (SEQ ID NO:213);
STIRPLPAIPRDTLLT (SEQ ID NO:214);
RSGRPLPPIPEVGHNV (SEQ ID NO:215);
IGSRPLPWTPDDLGSA (SEQ ID NO:216);
LAQRELPGLPAGAGVS (SEQ ID NO:217);
IPGRALPELPPQRALP (SEQ ID NO:218);
FVGRELPPTPRTVIPW (SEQ ID NO:219);
DPRSALPALPLTPLQT (SEQ ID NO:220); and
SPHDVLPALPDSHSKS (SEQ ID NO:221).

45. The method of claim 23 where the second molecule is a peptide that binds to the N terminal SH3 domain of Grb2, said peptide comprising an amino acid sequence selected from the group consisting of:
KWDSLLPALPPAFTVE (SEQ ID NO:224);
RWDQVLPELPTSKGQI (SEQ ID NO:225);
RFDFPLPTHPNLQKAH (SEQ ID NO:226);
RLDSPLPALPPTVMQN (SEQ ID NO:227);
RWGAPLPPLPEYSWST (SEQ ID NO:228);
YWDMPLPRLPGEEPSL (SEQ ID NO:229);
RFDYNLPDVPLSLGTA (SEQ ID NO:230);
TKKPNAPLPPLPAYMG (SEQ ID NO:231);
KWDLDLPPEPMSLGNY (SEQ ID NO:232);
YYQRPLPPLPLSHFES (SEQ ID NO:234);
YYRKPLPNLPRGQTDD (SEQ ID NO:235);

BNSDOCID: <WO___9730074A1_I_>

YFDKPLPESPGALMSL (SEQ ID NO:236);
YFSRALPGLPERQEAH (SEQ ID NO:237);
SLWDPLPPIPQSKTSV (SEQ ID NO:239);
SYYDPLPKLPDPGDLG (SEQ ID NO:240);
KLYYPLPPVPFKDTKH (SEQ ID NO:241); and
DPYDALPETPSMKASQ (SEQ ID NO:242).

46. The method of claim 23 where the second molecule is a peptide having an amino acid sequence selected from the group consisting of: SEQ ID NOs: 250-252, 254, 256-259, 261, 262, 264-266, 269-272, 275, 280, 281, 286-289, 291, 294, and 295.

47. The method of claim 23 where the second molecule is a peptide having an amino acid sequence selected from the group consisting of: SEQ ID NOs: 296-453.

48. A method of identifying a compound that affects the binding of a molecule comprising an SH3 domain and a ligand of the SH3 domain, the method comprising:

(a) contacting the SH3 domain and the ligand under conditions conducive to binding in the presence of a candidate compound and measuring the amount of binding between the SH3 domain and the ligand;

(b) comparing the amount of binding in step (a) with the amount of binding known or determined to occur between the molecule and the ligand in the absence of the candidate compound, where a difference in the amount of binding between step (a) and the amount of binding known or determined to occur between the molecule and the ligand in the absence of the candidate compound indicates that the candidate compound is a compound that affects the binding of the molecule comprising an SH3 domain and the ligand.

49. A kit comprising, in one or more containers:

(a) a purified first molecule comprising an SH3 domain;

(b) a purified second molecule that binds to the SH3 domain.

50. The kit of claim 49 wherein said second molecule comprises a peptide having an amino acid sequence selected from the group consisting of: SEQ ID NOs:107- 112, 114-126,

BNSDOCID: <WO__9730074A1_I_>

128-140, 142-159, 167, 168-172, 174, 176-195, 197-209, 211-221, 224-232, 234-237, 239-242, 250-252, 254, 256-259, 261, 262, 264-266, 269-272, 275, 280, 281, 286-289, 291, 294-453.

51. A purified peptide that binds to the SH3 domain of Src, said peptide comprising the amino acid sequence

$LX_1X_2RPLPX_3\psi PX_4X_5$ (SEQ ID NO:454)

where ψ represents aliphatic amino acid residues and X_1, X_2, X_3, X_4, and X_5 represent any amino acid; except that if X_3 = P, ψ = L, X_4 = P, and X_5 = P, then:

where X_1 = F, then X_2 is not H or R; or

where X_1 = S, then X_2 is not R, H, A, N, T, G, V, M, or W; or

where X_1 = C, then X_2 is not S or G; or

where X_1 = R, then X_2 is not T or F; or

where X_1 = A, then X_2 is not R, Q, N, S, or L; or

where X_1 = Q, then X_2 is not M; or

where X_1 = L, then X_2 is not R; or

where X_1 = I, then X_2 is not A; or

where X_1 = P, then X_2 is not P, W, or R; or

where X_1 = G, then X_2 is not S or R; or

where X_1 = T, then X_2 is not T.

52. A purified peptide that binds to the SH3 domain of Yes, said peptide comprising the amino acid sequence

$\psi X_1X_2RPLPX_3LPX_4X_5$ (SEQ ID NO:455)

where ψ represents aliphatic amino acid residues and X_1, X_2, X_3, X_4, and X_5 represent any amino acid; except that if X_3 = P, X_4 = P, and X_5 = P, then:

when ψ = L,

where X_1 = F, then X_2 is not H or R; or

where X_1 = S, then X_2 is not R, H, A, N, T, G, V, M, or W; or

where X_1 = C, then X_2 is not S or G; or

where X_1 = R, then X_2 is not T or F; or

where X_1 = A, then X_2 is not R, Q, N, S, or L; or

where X_1 = Q, then X_2 is not M; or

where X_1 = L, then X_2 is not R; or

where X_1 = I, then X_2 is not A; or

BNSDOCID: <WO__9730074A1_I_>

where X_1 = P, then X_2 is not P, W, or R; or
where X_1 = G, then X_2 is not S or R; or
where X_1 = T, then X_2 is not T; and
when ψ = P,
where X_1 = A, then X_2 is not R; or
where X_1 = S, then X_2 is not R or Y; or
where X_1 = M, then X_2 is not S; or
where X_1 = V, then X_2 is not G; or
where X_1 = R, then X_2 is not S; or
where X_1 = I, then X_2 is not R; and
when ψ = A,
where X_1 = A, then X_2 is not K; and
when ψ = V,
where X_1 = A, then X_2 is not C or Q; or
where X_1 = P, then X_2 is not P; and
when ψ = I,
where X_1 = G, then X_2 is not H; or
where X_1 = T, then X_2 is not S; or
where X_1 = R, then X_2 is not S.

BNSDOCID: <WO 9730074A1 | >

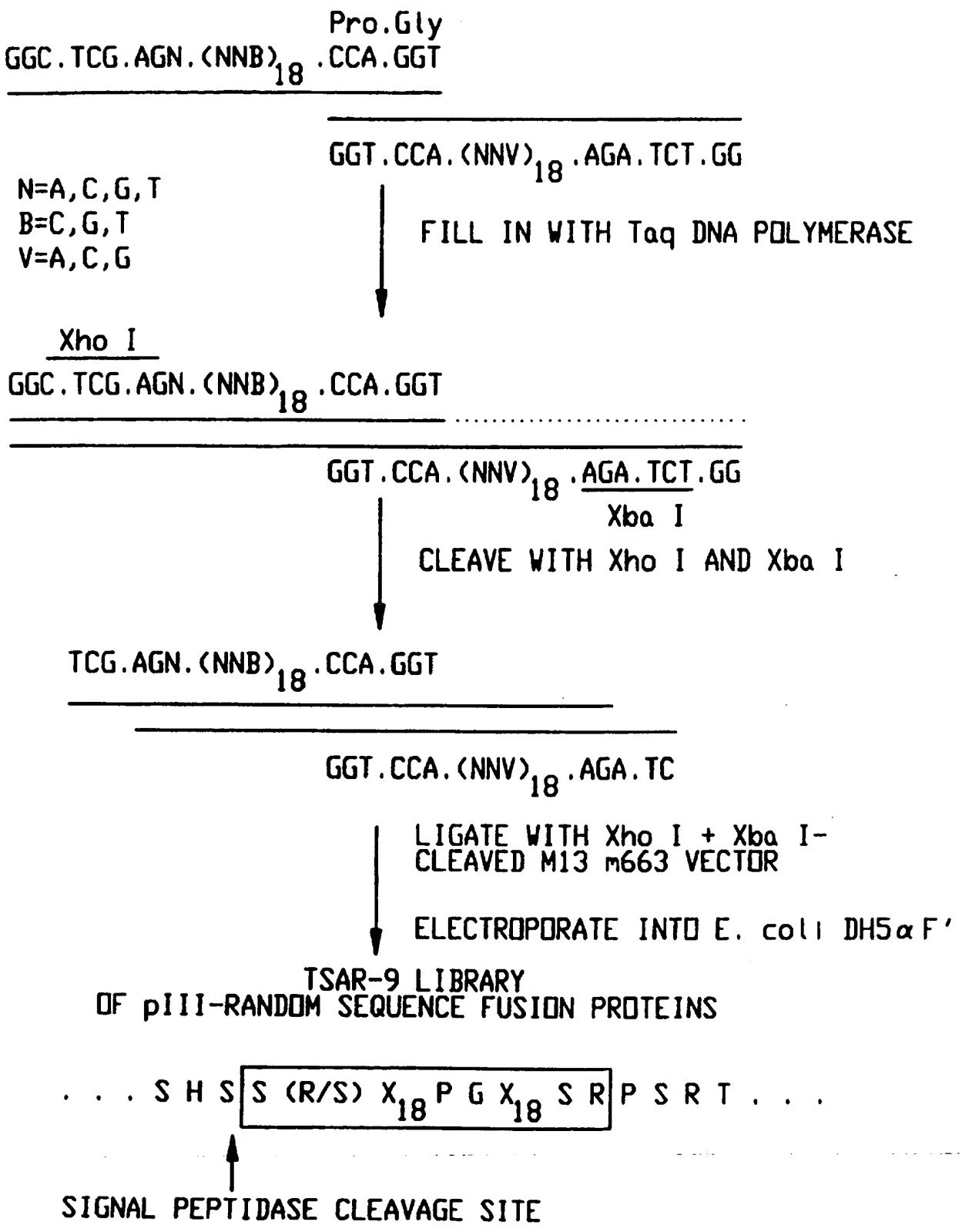

FIG.1

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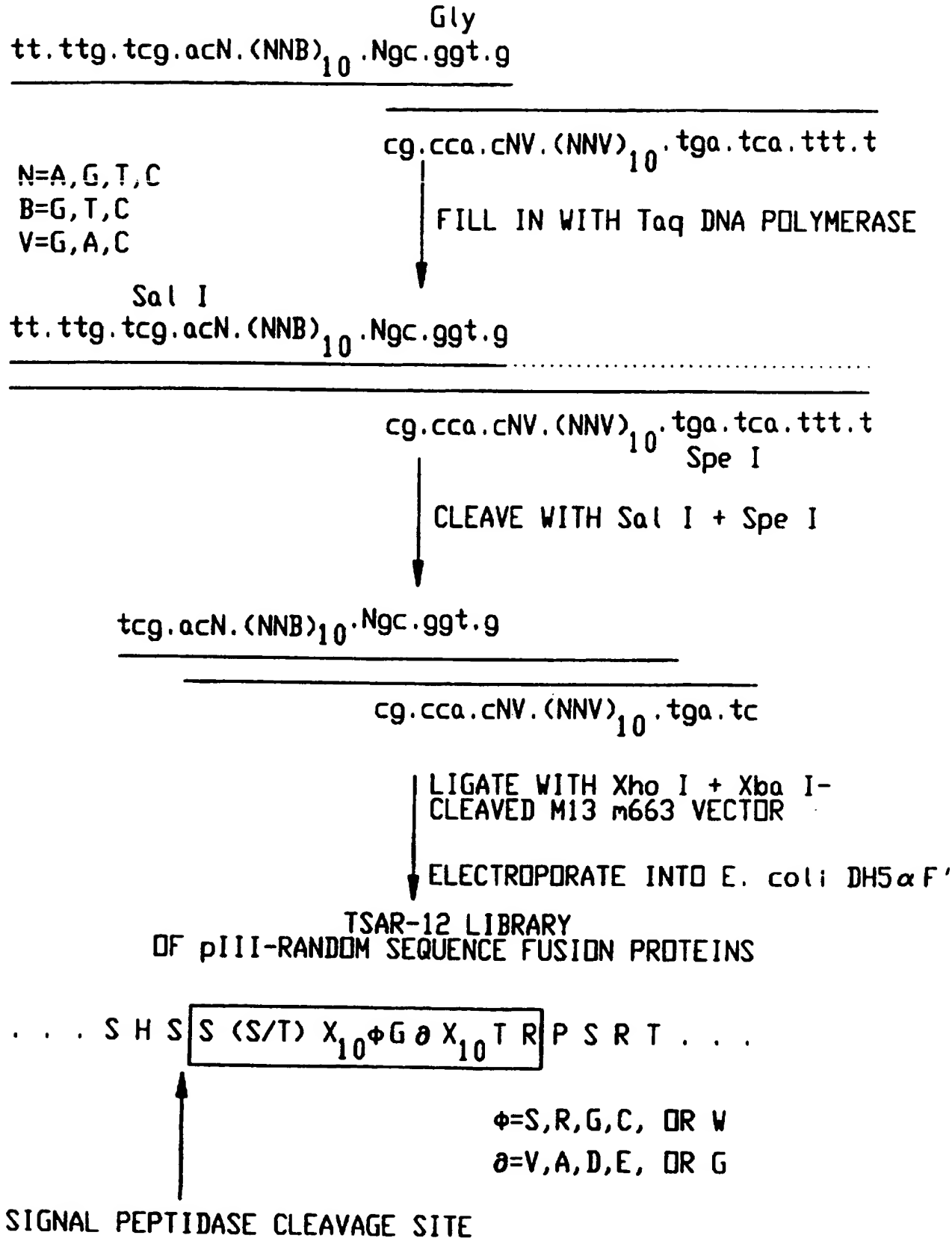

FIG.2

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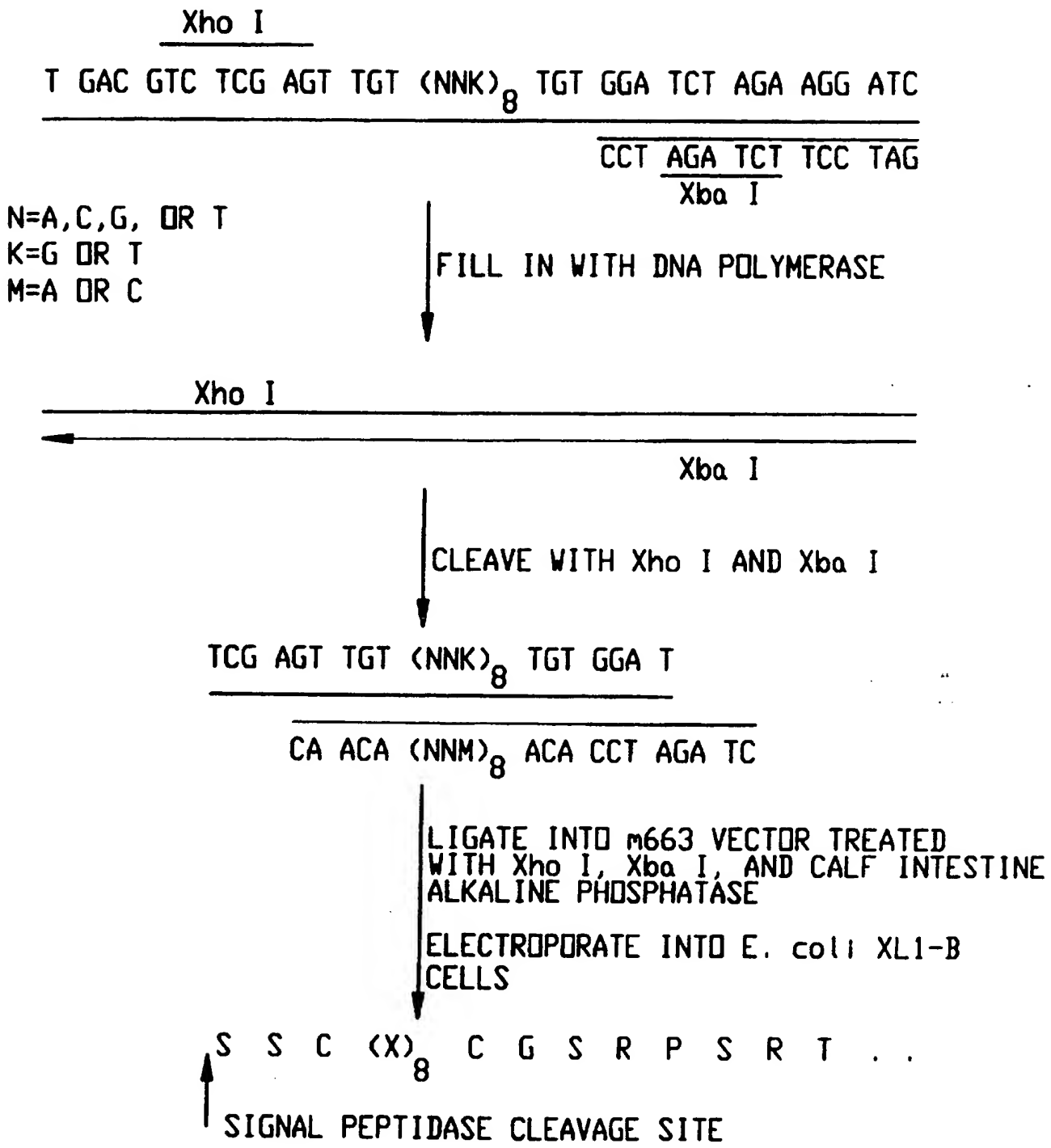

FIG.3

BNSDOCID: <WO___9730074A1_I_>

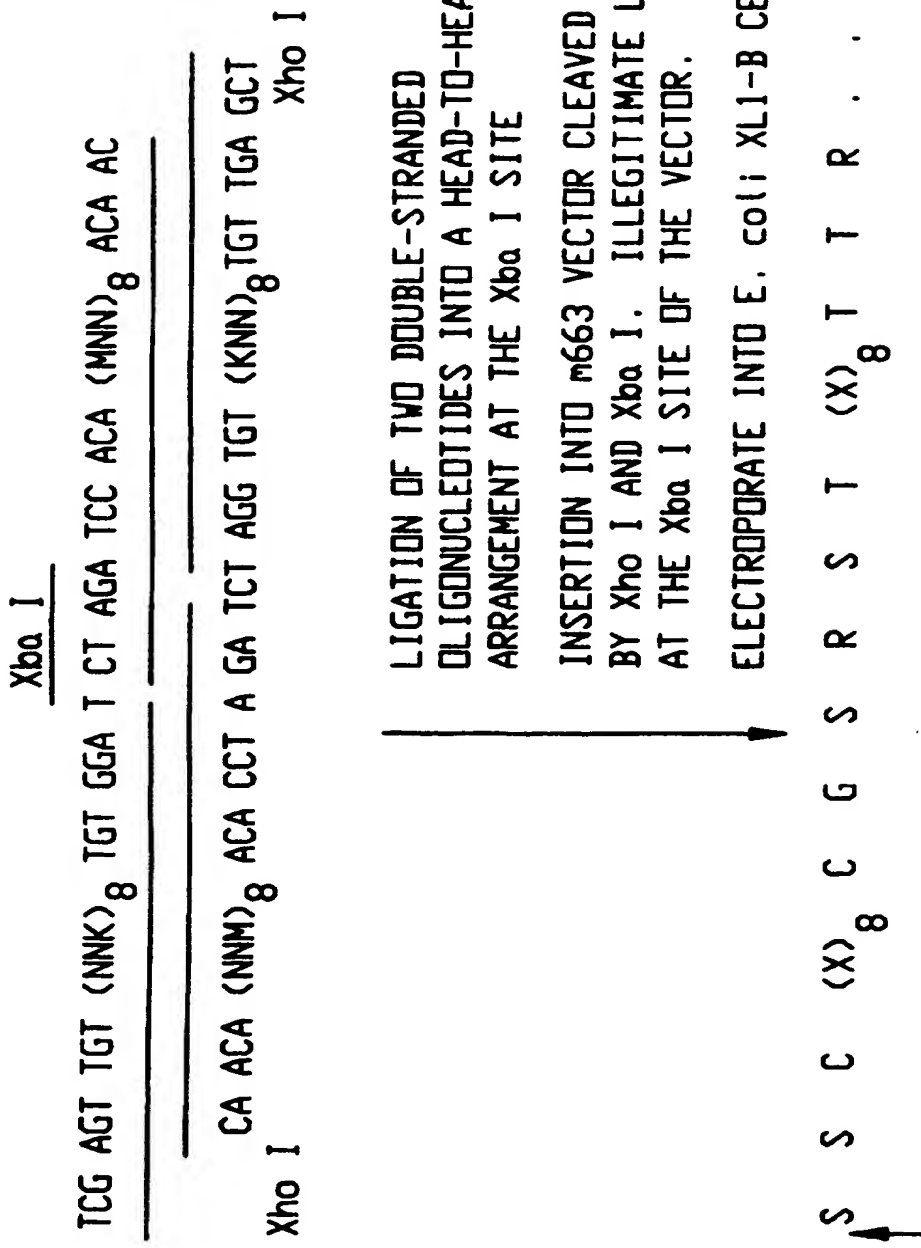

FIG.4

BNSDOCID: <WO___9730074A1_I_>

BNSDOCID: <WO__9730074A1_L>

SUBSTITUTE SHEET (RULE 26)

CLONE	SEQUENCE			FREQUENCY
T9.SRC3.2	SSFDQQDWDYSIAEKMHPIRPGF	RELPPLP	PSRASFGGGASRPSR	2
T12.SRC3.4	STNVWVTGSVIARGAQS	RPLPIPP	ETRPSR	1
T12.SRC3.6	STAPWGLRVAHEGGVLK	RPLPIPP	VTRPSR	1
T9.SRC3.4	SSSGYVVPKRLGDMREYNAHPGLHVPPN	SPLPPLP	THLQSSRPSR	1
T9.SRC3.6	SSRGEGNNIISSRPFLSNSDPGVSNKLTGR	GPLPPLP	NDSRPSR	2
T12.SRC3.7	STAVSFRFMPGGGGAFYST	RPVPPIT	RPSRT	1
T12.SRC3.5	STAHSLWDWGTFSGVSHKS	RLPPLPT	RPSRT	1
T9.SRC3.7	XPGYARIVSYRF	RALPSPP	SASRPSR	1
T12.SRC3.3	STNDVDWMHMWNSGGPH	RRLPPTP	ATRPSR	4
T9.SRC3.5	SSDNWARRVHASELIYTDLSPGILLAQ	RQLPPTP	GRDPSHSRPSR	1
T9.SRC3.1	SSESPLMYNRVGALQSLTSVPGSMMHFALQ	RRLPRTP	PPASRPSR	5
T12.SRC3.2	STRWSHSWPGYVGGANPSPAT	RPLPTRP	SRTVESC	19
T9.SRC3.3	SRYNDLGTRPVSEVIKYDYFPGYSQHVITPDGSYST	RPLPSRP	SRTVESC	2
T9.SRC3.8	XPG	RILLLPS	EPRTFYNYGHDSRPSR	1
T12.SRC3.1	STMYGVSWLSSGSGGILA	PPVPPRN	TRPSR	25
	Consensus	RPLPPLP		
R8C.YES3.6	SSCTEKTVSGWCGSRST	RPLPILP	RTTRPSR	1
R8C.YES3.5	SSCMLPTDGWQCGSRSTP	RPLPMLP	TTRPSR	1
R8C.YES3.3/SRC3.1	SSCDGTQFRLNCGSRSTN	RPLPMIP	TTRPSR	3
R8C.YES3.1/SRC3.2	SSCMQGQAGLKCGSRST	RPLPSLP	ITTRPSR	7
R8C.YES3.7	SSCYREKDTWGCGSRSTS	RPLPSLP	TTRPSR	2
R8C.YES3.2	SSCLFEQGAGTCGSRST	RSLPPLP	PTTRPSR	2
R8C.YES3.4	SSCDHTLGLGWCGSRST	RQLPIPP	TTTRPSR	1
R8C.YES3.10	SSCDTGRIAPGCGSRSTP	RPLPLIP	TTPRSTNLNLTSTTRPSR	2
R8C.YES3.8	SSCGLDNAAKTCGSRST	RPLPPTP	LTTRPSR	2
R8C.YES3.9	SSCSRAHETEMCGSRST	RPQPPPP	ITTRPSR	2
	Consensus	RPLPPLP		

FIG.5

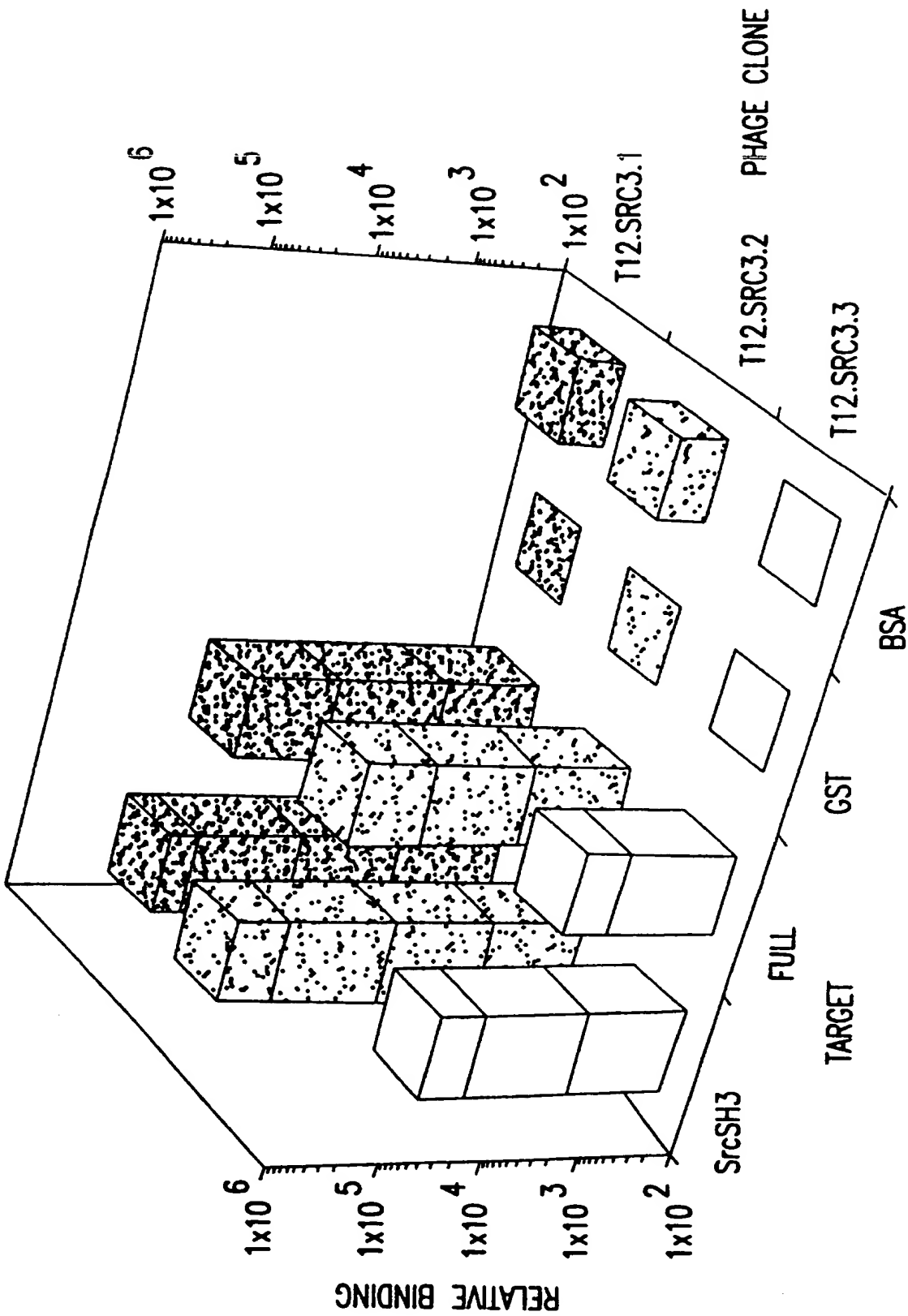

FIG.6

BNSDOCID: <WO___9730074A1_I_>

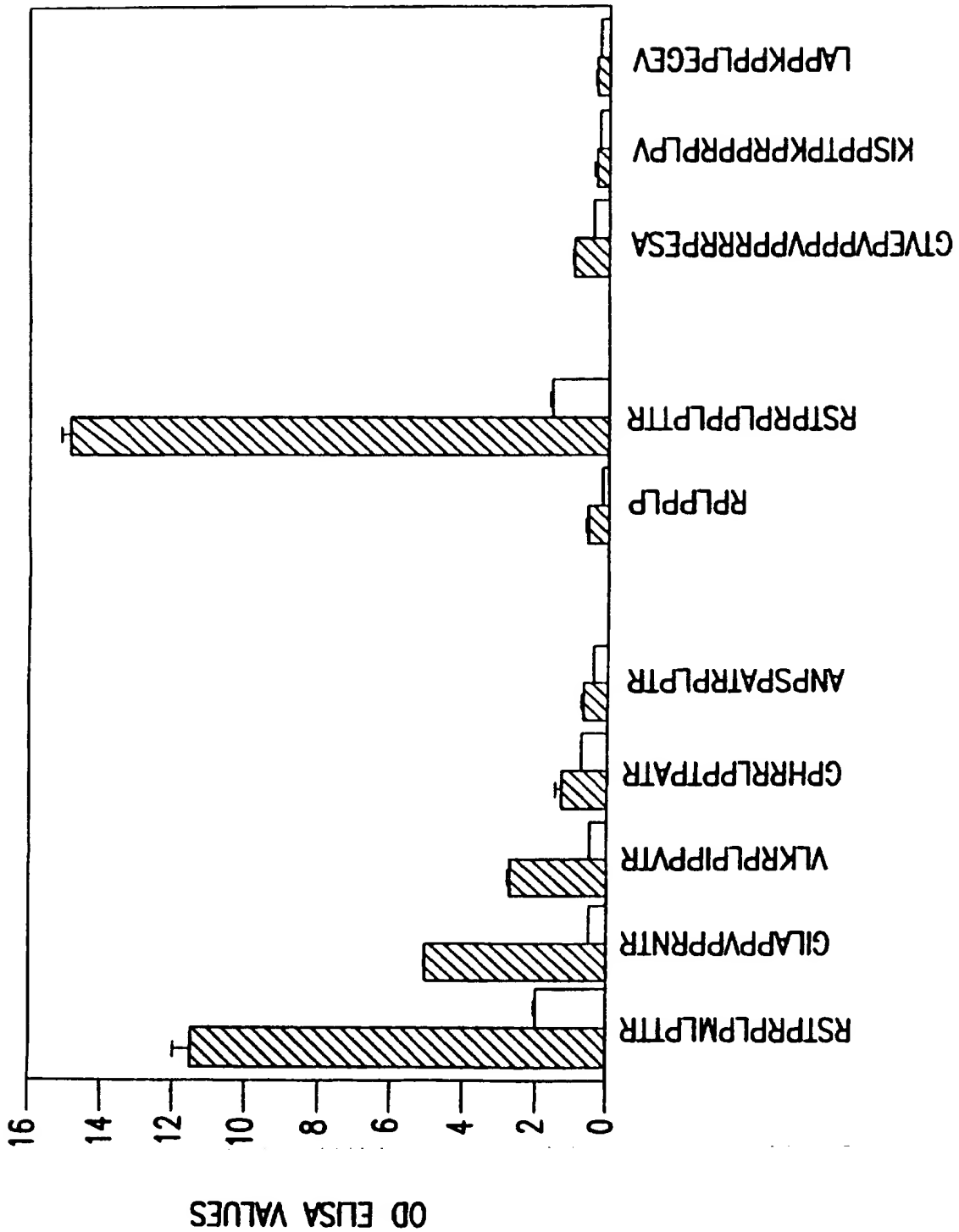

FIG.7

BNSDOCID: <WO___9730074A1_I_>

ENSDOCID: <WO 9730074A1_L>

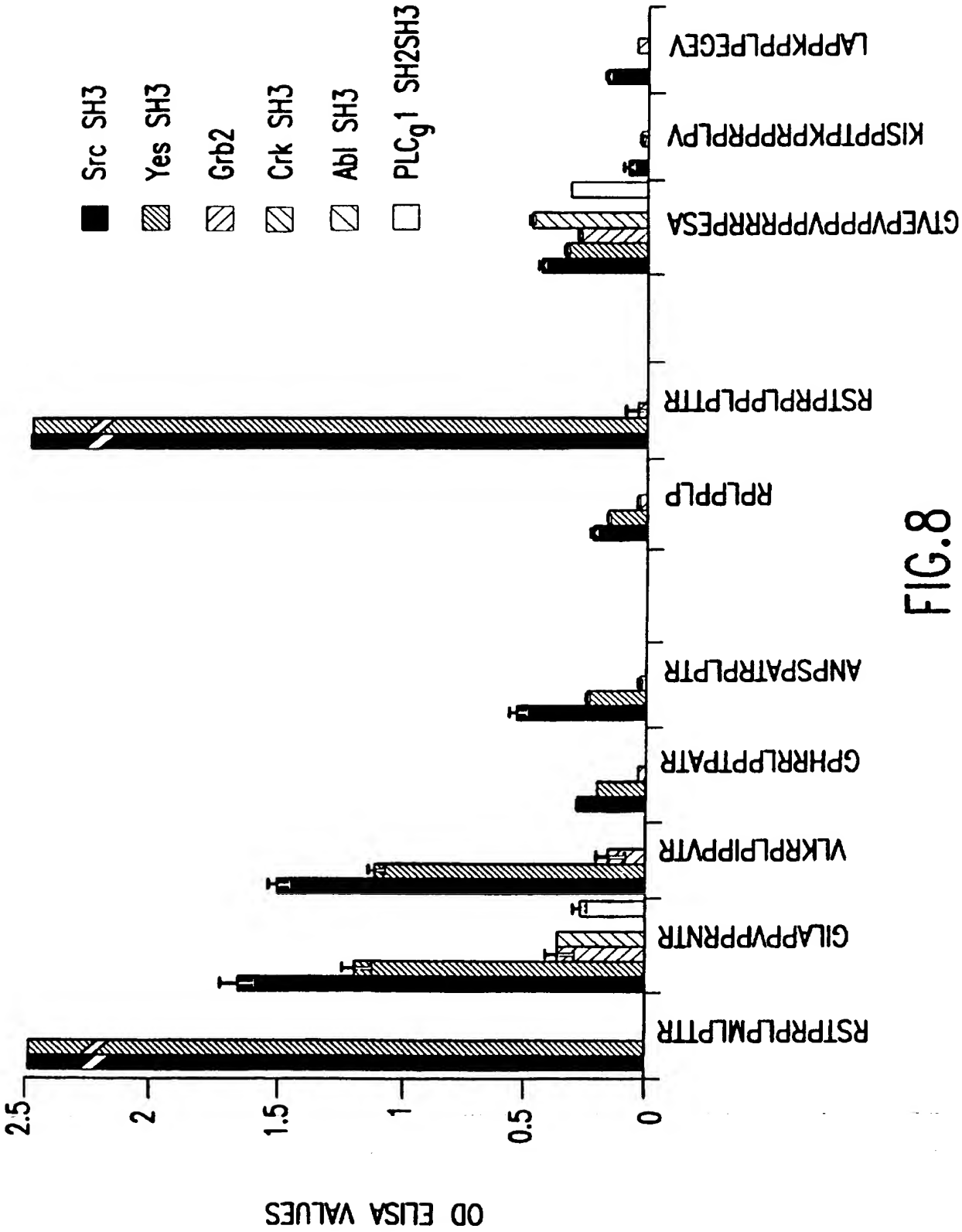

FIG.8

BNSDOCID: <WO___9730074A1_I_>

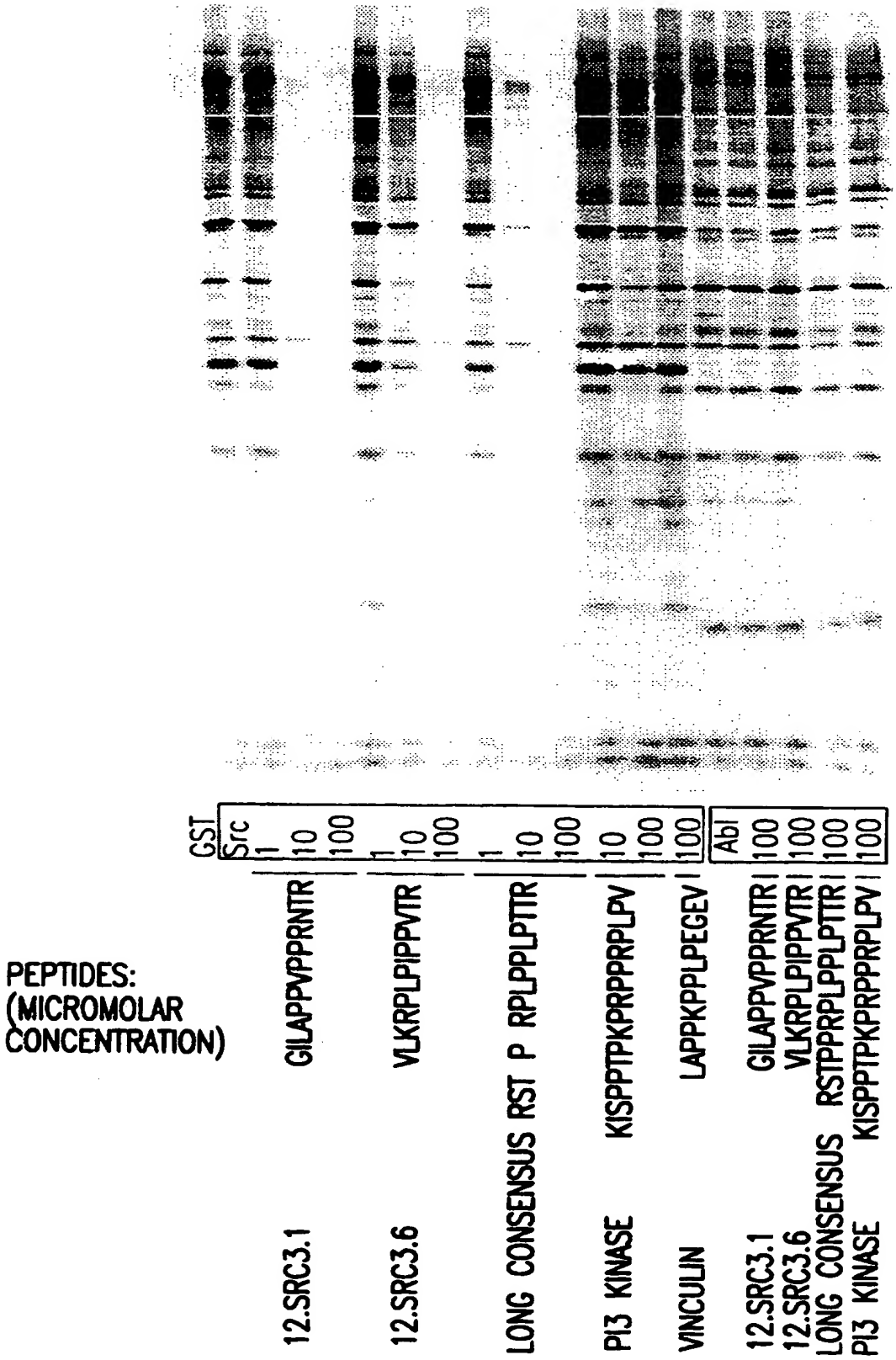

FIG.9

BNSDOCID: <WO___9730074A1_I_>

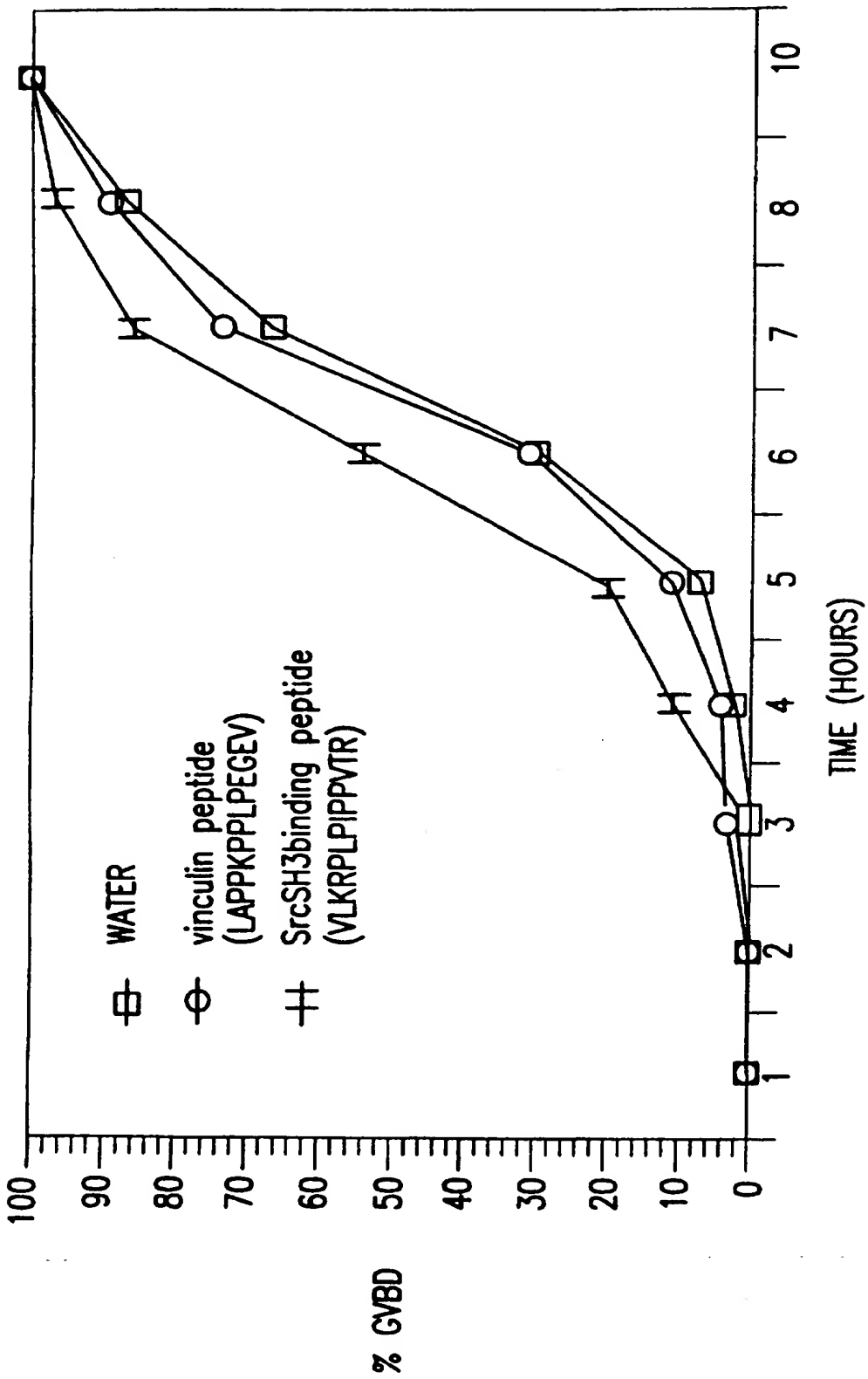

FIG.10

BNSDOCID: <WO___9730074A1_I_>

FIG. 11A

FIG. 11B

BNSDOCID: <WO___9730074A1_I_>

FIG. 11C

FIG. 11D

BNSDOCID: <WO___9730074A1_I_>

FIG.12

BNSDOCID: <WO___9730074A1_I_>

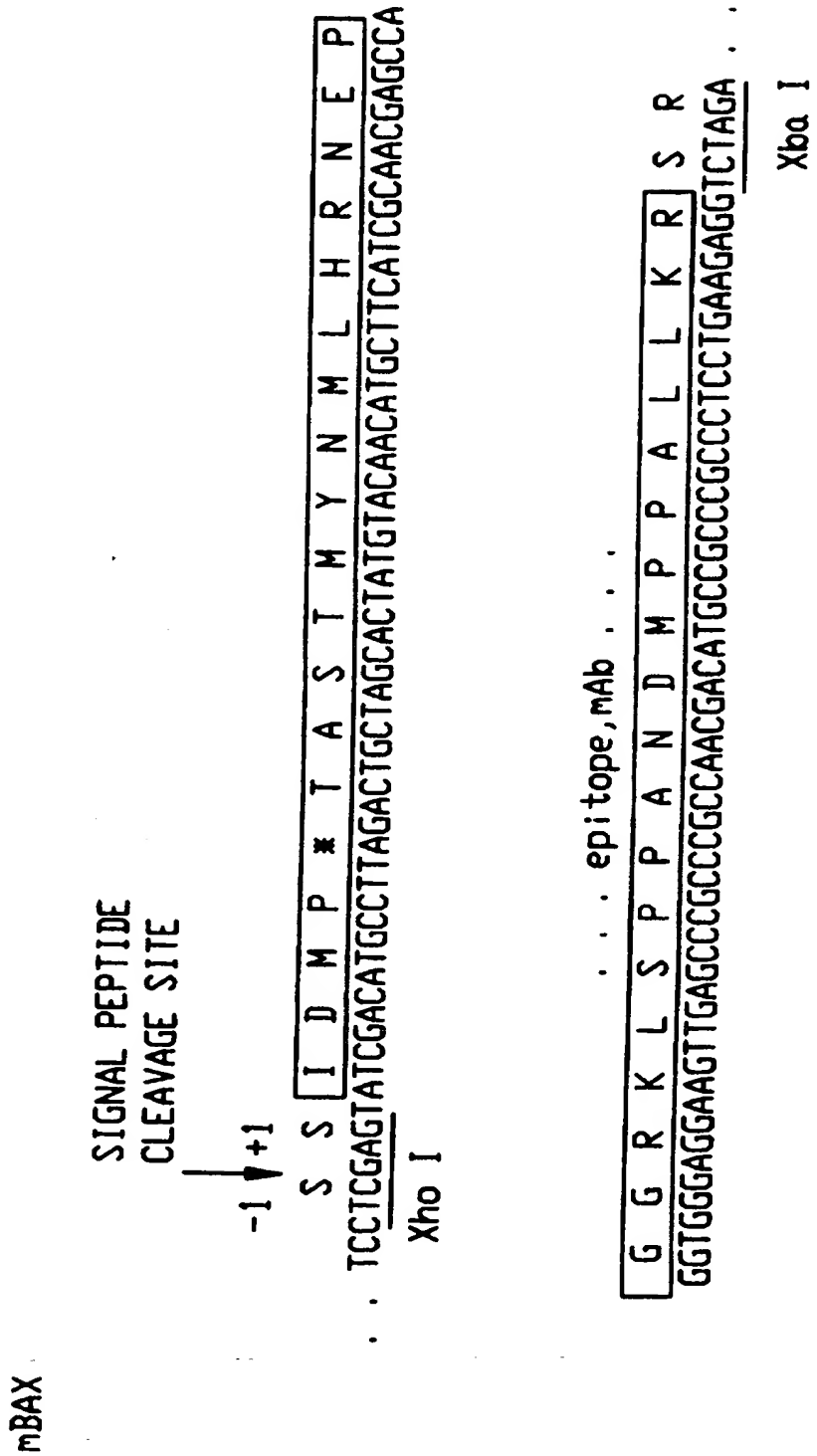

FIG.13

BNSDOCID: <WO___9730074A1_I_>

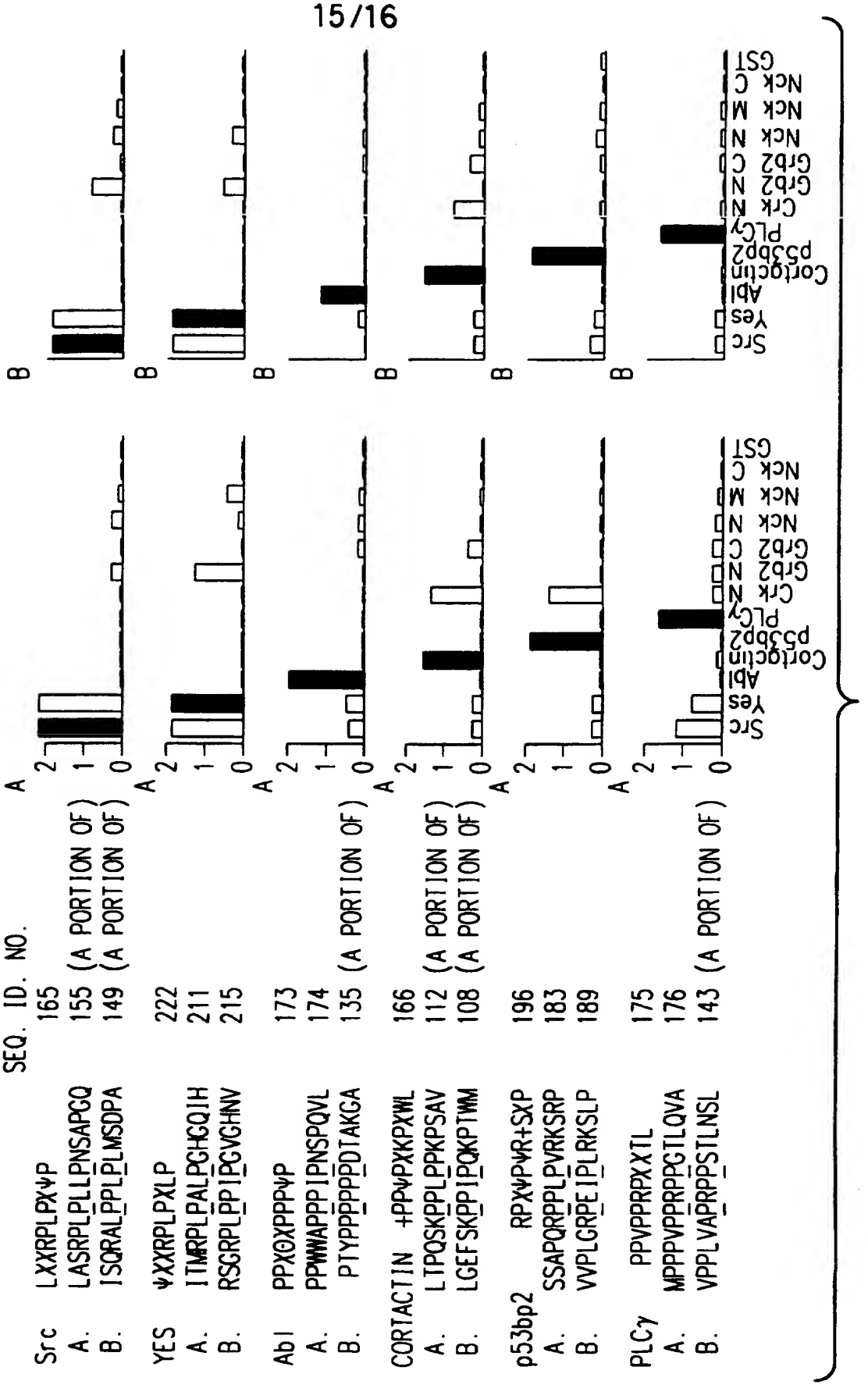

FIG.14

BNSDOCID: <WO 9730074A1 >

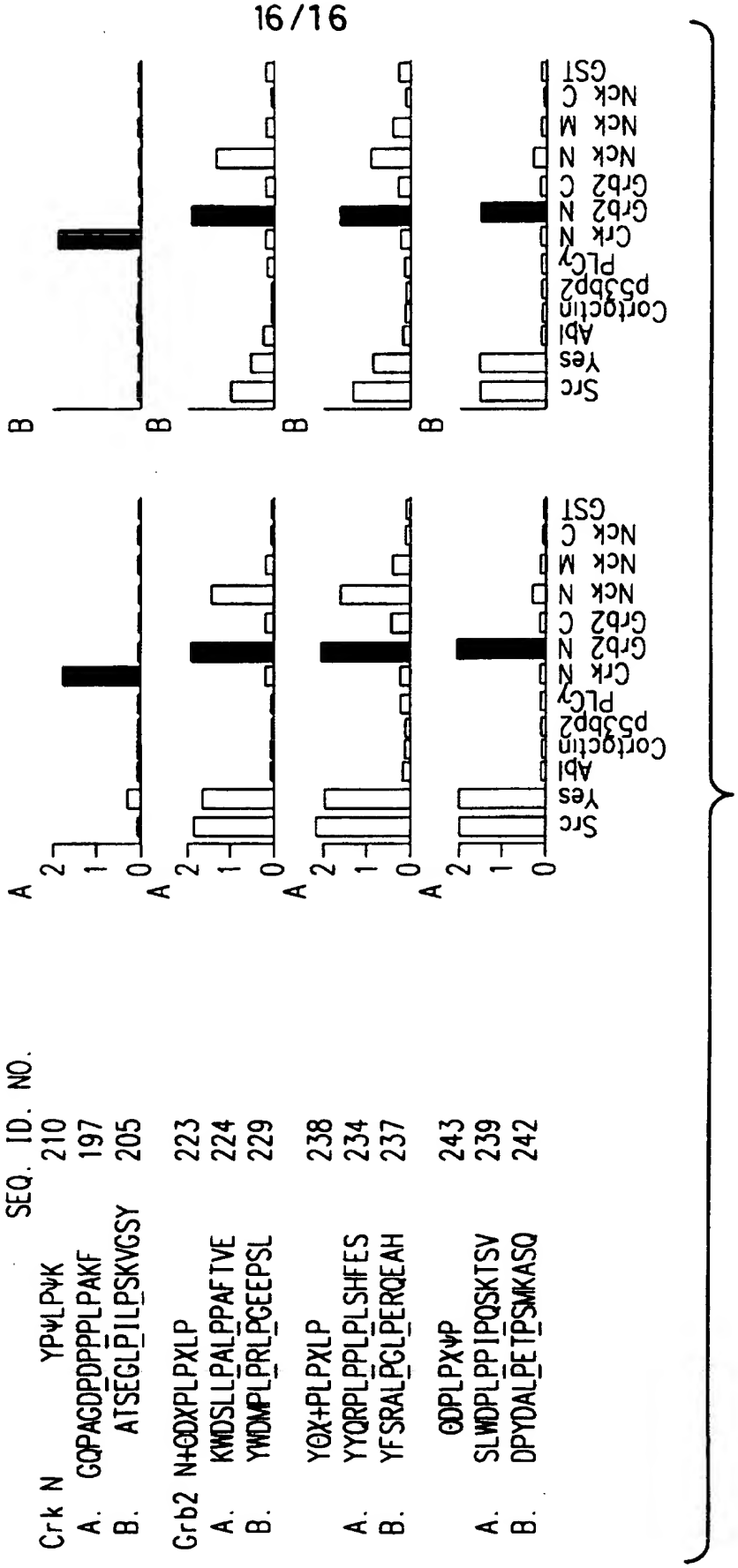

FIG.14A

BNSDOCID: <WO___9730074A1_I_>

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/02298

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :Please See Extra Sheet.
US CL :Please See Extra Sheet.
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 530/350, 324, 325, 326, 327, 328, 329, 828; 435/7.1, 7.2, 7.23, 7.8

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, Medline, Inpadoc

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	OKAMURA et al. p80/85 Cortactin Associates with the Src SH2 Domain and Colocalizes with v-Src in Transformed Cells. JOURNAL OF BIOLOGICAL CHEMISTRY November 3, 1995. Vol. 270, No. 44, pages 26613-18, see entire document.	1, 5, 12, 23-26, 30, 37, 46-50
X	Feller et al. Cellular proteins vinding to the first Src homology 3 (SH3) domain of the proto-oncogene product c-Crk indicate Crk-specific signaling pathways. ONCOGENE 20 April 1995. Vol. 10, No. 8, pages 1465-1473, see entire document.	2, 9, 13, 18, 23-25, 27, 34, 38, 43, 46-50

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 11 JUNE 1997	Date of mailing of the international search report 1 JUL 1997
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer Jeffrey Stucker Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

BNSDOCID: <WO___9730074A1_I_>

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/02298

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	RICKLES et al, Identification of Src, Fyn, Lyn, PI3K and Abl SH3 domain ligands using phage display libraries. EMBO JOURNAL. 01 December 1994. Vol. 13, No. 23, pages 5598-5604, see entire document.	3, 4, 6, 14, 16, 23-25, 28, 29, 31, 39, 41, 46-51
X	LEE et al, Association of CD45 with Lck and components of the Ras signallling pathway in pervanadate-treated mouse T-cell lines. ONCOGENE. 18 January 1996. Vol. 12, No. 2, pages 253-263, see entire document.	7, 15, 23-25, 32, 40, 46-50
X	SUDOL. Yes- Associated Protein (YAP65) is a proline-rich phosphoprotein that binds to the SH3 somain of the Yes proto-oncogene product. ONCOGENE. August 1994. Vol. 9, No. 8, pages 2145-2152, see entire document.	10, 19, 23-25, 35, 44, 46-50, 52
X	D'AMBROSIO et al. The role of Grb2 in the growth and transformation of mouse embryo cells. ONCOGENE. 18 January 1996. Vol. 12, No. 2,pages 371-378, see entire document.	11, 20, 23-25, 36, 45-50
X, P	SPARKS et al. Distinct ligand preferences of Src homology 3 domains from Src, Yes, Abl, Cortactin, p53bp2, PLCgamma, Crk, and Grb2. PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF THE UNITED STATES OF AMERICA. 20 February 1996. Vol. 93, No. 4, abstract only.	3, 6, 8, 14, 17, 23-25, 28, 31, 33, 39, 42, 46-50

BNSDOCID: <WO___9730074A1_I_>

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/02298

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☒ Claims Nos.: 21 and 22
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

The computer sequence listing was defective. Therefore, the computer readable sequence data was not entered. See attached raw sequence listing error report. Claims 21 and 22 are directed only to specific seqences, and therefore, could not be searched without the computer sequences.

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

BNSDOCID: <WO___9730074A1_I_>

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/02298

A. CLASSIFICATION OF SUBJECT MATTER:
IPC (6):

C07K 5/04, 07/04, 07/06, 07/08, 14/00, 14/435, 14/705; G01N33/53, 33/567

A. CLASSIFICATION OF SUBJECT MATTER:
US CL :

530/350, 324, 325, 326, 327, 328, 329, 828; 435/7.1, 7.2, 7.23, 7.8

BNSDOCID: <WO___9730074A1_I_>

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